Chapter 17

Application of Quartz Crystal Microbalance with Dissipation Monitoring Technology for Studying Interactions of Poxviral Proteins with Their Ligands

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Abstract

Poxviruses are one of the most complex of animal viruses and encode for over 150 proteins. The interactions of many of the poxviral-encoded proteins with host proteins, as well as with other proteins, such as transcription complexes, have been well characterized at the qualitative level. Some have also been characterized quantitatively by two hybrid systems and surface plasmon resonance approaches. Presented here is an alternative approach that can enable the understanding of complex interactions with multiple ligands. The example given is that of vaccinia virus complement control protein (VCP). The complement system forms the first line of defense against microorganisms and a failure to appropriately regulate it is implicated in many inflammatory disorders, such as traumatic brain injury, Alzheimer's disease (AD), and rheumatoid arthritis. The complement component C3 is central to the complement activation. Complement regulatory proteins, capable of binding to the central complement component C3, may therefore effectively be employed for the treatment and prevention of these disorders. There are many biochemical and/or immunoassays available to study the interaction of proteins with complement components. However, protocols for many of them are time consuming, and not all assays are useful for multiple screening. In addition, most of these assays may not give information regarding the nature of binding, the number of molecules interacting with the complement component C3, as well as kinetics of binding. Some of the assays may require labeling which may induce changes in protein confirmation. We report a protocol for an assay based on quartz crystal microbalance with dissipation monitoring (QCM-D) technology, which can effectively be employed to study poxviral proteins for their ability to interact with their ligand. A protocol was developed in our laboratories to study the interaction of VCP with the complement component C3 using Q-sense (D-300), equipment based on QCM-D technology. The protocol can also be used as a prototype for studying both proteins and small-sized compounds (for use as anti-poxvirals) for their ability to interact with and/or inhibit the activity of their ligands.

Key words: Poxviral protein–ligand interaction, Quartz crystal microbalance with dissipation monitoring, VCP

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1. Introduction

Poxviral outbreaks are common in different parts of the world (1–4). These viruses secrete many proteins which help these viruses in establishing infections in their host. Most of these proteins play an important role in the pathogenesis of these viruses by interacting with the immune system and/or by evading the immune response of the host. Study of interaction of these viral proteins with the proteins of the immune machinery of the host might prove beneficial for developing an effective treatment strategy to treat or prevent the progression of the pathogenesis of these viral infections. Some of the poxviruses also secrete complement regulatory molecules, which evade the complement-mediated immune response. These complement regulatory molecules may also be of great help in the treatment and prevention of a number of diseases, where the complement system is up-regulated. Vaccinia virus is one of the viruses belonging to poxviral family that secretes a complement control protein. The vaccinia virus complement control protein (VCP) is known to regulate the complement system (5–7), and by virtue of its complement regulatory activity, it has been shown to be effective in rodent models of traumatic brain injury (8), Alzheimer's disease (AD) (9, 10), spinal cord injury (11), and atherosclerosis (12), where up-regulation of the complement system is evident. VCP has previously been shown to interact with the C3b and C4b components of the complement system using surface plasmon resonance technology (SPR (13)). It has recently also been shown to bind to the complement component C3 using a relatively new quartz crystal microbalance with dissipation monitoring technology (QCM-D; (14)). The protocols in the aforementioned article are explained in greater detail here in this chapter.

The complement component C3 is one of the proteins of the complement system that has been found to be up-regulated in several neuroinflammatory disorders, such as AD. The level of C3 is known to be elevated in AD with mild to severe clinical symptoms with a low level of expression of complement regulatory molecules (15). Therefore, some believe that the activated complement components, especially C3 which is central to complement activation, need to be regulated in such disease states. The discovery and development of agents that prevent the detrimental effects of these complement components might have therapeutic potential. Techniques to identify compounds with an ability to bind to C3 and inhibit the activation of complement would be useful. However, immunoassays and other biochemical assays required to study the protein–protein interactions are costly and time consuming. Also, most of these assays do not give the information regarding the nature of binding of the two interacting moieties, number of molecules binding, as well as kinetics of binding. In addition, the assays

need to be customized if one wants to study the interaction of small-sized compounds with the proteins.

There are many techniques available for protein adsorption or interaction (16). In order to carry out the protein binding study, we used QCM-D-based Q-sense (D-300), a Swedish-based technology thoroughly studied and developed for the protein adsorption studies at Chalmer's University of Technology, Goteberg, Sweden (17). QCM-D is a rapidly advancing technology used to study the protein–surface interactions and ligand–receptor interactions. This technology has recently been used to study the change in conformation of the protein molecules during co-precipitation (18), deposition kinetics of nanoparticles (19), screening membrane-active antimicrobial peptides (20), and changes in viscoelastic properties during the phase transitions between the two states of cowpea chlorotic mottle virus (21).

The QCM-D technique offers the advantage of real-time monitoring, rapidity, simplicity, sensitivity and economy, compared to the other routinely used techniques. The potential of QCM as a biosensor and its sensitivity was found to be comparable to that of SPR (22, 23), a commonly used technique for protein binding studies. Using bare gold crystals of QCM, it was shown to be as sensitive as ELISA when compared using gp41-based HIV-ELISA (24). It offers an advantage of real-time monitoring and rapidity (10 min to 2 h versus several hours to 1–2 days in ELISA) without losing sensitivity. It does not involve labeling of protein molecules and, therefore, decreases concern about possible changes in conformation when protein needs to be labeled. It also takes into account dissipation (D) values or the ratio of change in dissipation to change in frequency (dD/dF) values at the same time point. These values are explained later in the text, and can be used to study the nature (rigid vs. reversible) as well as kinetics of protein binding. The many advantages of QCM-D technology over the other routinely employed techniques as discussed above led us to employ Q-sense D-300 (Q-sense) to study the interaction of VCP with complement components.

QCM-D has previously been employed to study the interaction of the complement system with biomaterials (25). The technique was also used to study the interaction of the complement components with complement inhibitory molecules. QCM-D was compared to the ELISA technique to show that biomaterial surfaces activate the complement system and this activation can be inhibited by complement regulatory molecules, such as factor H (26). Using QCM-D, it was also shown that the polystyrene surface activates the alternative pathway (AP) of complement activation. C3 deposited on the polystyrene surface can form C3 convertase and activates the AP of complement activation (25). Thus, Q-sense based on QCM-D may be employed to investigate the complement regulatory molecules with an ability to bind the

activated form of C3. In this chapter, we describe the application of the technique of using the polystyrene sensor (PS) crystals to study the interaction of VCP with the complement component C3 adsorbed on PS crystals.

The overall objective of applying the QCM-D technique is to specifically compare the interactions of VCP and a truncated version of VCP (tVCP) with C3 on the PS surfaces.

Objectives

(a) Compare the nature of binding of VCP and tVCP with that of C3 using PS.

(b) Compare the adsorption kinetics of interaction of VCP and tVCP with PS.

(c) Compare the interaction of C3 with the aforementioned surfaces as well as with VCP and tVCP adsorbed onto these surfaces.

2. Materials

1. Q-sense, model D-300.
2. Polystyrene sensor crystals (PS; Q-sense).
3. Piranha solution: 1:1:5 of HCl:H_2O_2 (30%):H_2O.
4. 1% Hellmanex-II solution (a cleaner concentrate for cuvette washing).
5. Nitrogen gas.
6. UV chamber (e.g., Bioforce, nanoscience) (see Note 1).
7. Phosphate-buffered saline (PBS).
8. Purified proteins of interest, passed through a 0.22-μM filter and degassed prior to use (see Note 2).

3. Methods

3.1. Basic Principles of QCM, Terminologies Used, and Description of Q-Sense

QCM is used to measure the interaction between protein or adsorbing molecules and that of the surface as well as with each other, employing a piezoelectric sensor crystal. For the QCM studies, this piezoelectric sensor crystal is coated with a very thin layer of gold on both the sides. The bottom surface serves as electrodes to which AC voltage is applied. This results in oscillation of the sensor crystal at its resonant frequency, *f*. This inherent *f* of the quartz crystal changes upon adsorption of a thin layer of an adsorbing moiety (AM). In QCM, this change in frequency (d*F*) is correlated with the change in mass (ΔM or d*M*). The amount of a substance

deposited on the crystal surface is determined from this change in frequency using Sauerbrey's equation as discussed by Höök in his thesis and the references mentioned therein (17). As discussed by Höök, the Sauerbrey's equation is based on the assumptions that the AM should be rigidly deposited on the crystal, distributed evenly, and the added mass is smaller than the weight of the crystal. "Fit analysis" as outlined in the manual of q-sense should be carried out to check whether the experiments follow the assumptions based on the Sauerbrey's equation.

Dissipation factor or D factor in QCM-D gives information regarding the energy dissipated during one oscillation after the adsorption of the AM on the crystal. The ratio dD/dF in turn gives information regarding the rigidity of binding at the interfaces (27–29). Further information on this factor can be obtained from the research articles published in the literature (14, 27–31).

3.2. Q-Sense: Practical Considerations (Q-Sense Reference Manual (14, 17)

1. The basic layout of the Q-sense (model D-300) is as shown in Fig. 1. It consists of four basic components: sensor crystal, measurement chamber, electronic unit, and acquisition software.
2. Inside the chamber (Fig. 1b), the PS crystals (Fig. 1d) are operated in thickness shear mode at 5 MHz frequency and are mounted as per the instructions in the manual.
3. The smooth surface of the crystal is used for the measurements, and the opposite side of the crystal is connected to the A/C supply of the electrical unit.
4. The measurement chamber also consists of a sample holder (Fig. 1c), where the solution of the AM is kept. The sample holder can be directed to either a "temperature loop" or to the "sensor crystal" using a controller knob.

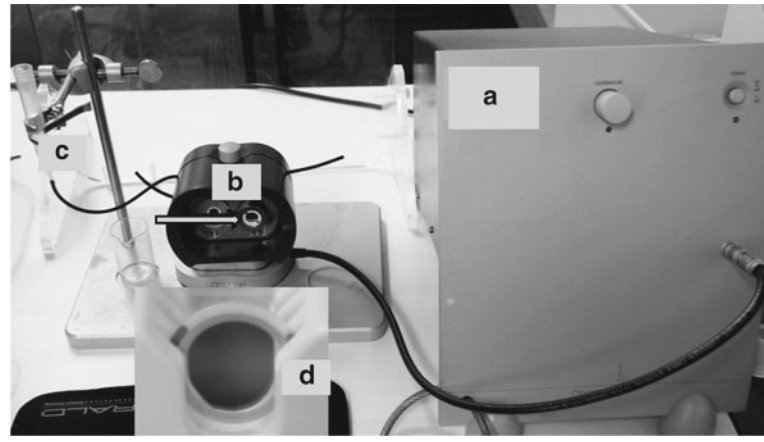

Fig. 1. The Q-sense (D-300) apparatus and its components. Shown are the electronic unit (**a**), measurement chamber (**b**), and the sample holder (**c**). The PS sensor crystal mounted on the 'O' ring of the measurement chamber is shown by an *arrow near* (**b**) and the smooth surface of the sensor crystal where AMs are adsorbed is shown in the *inset* (**d**).

5. The measurement chamber is connected to a computer to measure the changes in f and D values at different overtones. These changes in f and D values are monitored online using q-soft/QTools software supplied with the equipment and are used for further analysis.

3.3. Explanation of Terminology

1. AM1 = AM bound to the surface of the sensor crystal. Therefore, AM1 is indicated by putting the first letter of the surface (ps = polystyrene) in lower case, next to AM1. For example, when the AM is VCP or C3 and when the AM is bound to the surface of the sensor crystal, the abbreviations are VCPps and C3ps.
2. AM2 = a second adsorbing moiety that is bound to AM1. For example, when C3 is bound to VCPps, the abbreviation would be C3-VCPps or when VCP is bound to C3ps, the abbreviation would be VCP-C3ps.
3. dF_{30} and dD_{30} refer to dF and dD values after 30 min, respectively. dF_{30} and dD_{30} values for AMs are calculated as follows.
   (a) $dF_{30} = dF$ at 30 min $- dF_{initial}$, where $dF_{initial}$ refers to the baseline or initial f or D values prior to the adsorption of AMs.
   (b) dF_{fin} or dD_{fin} values indicate the final dF and dD values recorded after washing the surface with PBS after the adsorption is over. dF_{fin} = Final dF (or dD) values after washing with PBS $- dF_{initial}$ (or $dD_{initial}$).
   (c) Using these dD and dF values, calculate dD/dF ratios to get the information regarding the rigidity (affinity) or viscoelasticity of binding.
   (d) Calculate the number of molecules of AM adsorbed (n) onto the crystal surface or onto the other AM using Avogadro's formula ($n = (6.022 \times 10^{23} \times$ mass adsorbed (ng/cm^2))/molecular weight of the AM).

3.4. Studying Interactions of Poxviral Proteins with Their Ligands Using QCM-D Technology

1. Just before starting the experiment and after completion of the experiment, clean the surface of the crystal and the chamber according to the protocol provided in the Q-sense instruction manual by washing the PS crystal with piranha solution and washing the chamber with 1% Hellmanex-II solution (see Note 3).
2. After each washing step, dry the sensor crystals and chamber using nitrogen gas.
3. Expose the sensor crystal to UV light for 5–10 min to destroy impurities on the sensor surface (see Note 1).
4. Ensure that resonant frequencies of the crystals match the values provided in the instruction manual (see Notes 4 and 5).
5. Obtain baseline readings using PBS before starting each experiment as well as prior to each adsorption (see Note 6).
6. Prior to the adsorption of AM1, obtain a baseline for the resonance frequency of the crystal by adding PBS to the surface.

Table 1
The outline of the Q-sense experiments

Sr	Surface	AM 1	AM 2
1	PS	VCP	C3
2	PS	C3	VCP
3	PS	tVCP	C3

Sr serial number, *PS* polystyrene, *AM* adsorbing moiety (AM1, AM2)

7. Add PBS until there is no further drop in the frequency. An example of the surfaces, AMs, and the sequence of adsorbing moieties is shown in Table 1.
8. Adsorb AM (e.g., VCP, tVCP, or other poxviral protein or C3) on the PS crystal surface. This is AM1 (see Notes 7–9).
9. After adsorption of the first AM on the sensor crystal for a particular period of time (approximately 30-50 min), wash with PBS to remove any unadsorbed molecules.
10. Add the second AM (AM2) to the sensor surface to adsorb to AM1 and allow adsorption for the same period of time (approximately 30–50 min) (see Notes 7 and 8).
11. Wash with PBS to remove any unadsorbed molecules.
12. Measure the changes in f and D values at different overtones using QTools software.
13. Analyze the data captured using q-soft/QTools software (see Notes 10–12).
14. Confirm whether the adsorption of the AM follows the Sauerbrey's equation using fit analysis and plot the data as shown in Fig. 2.
15. Calculate the dD/dF ratios to get information regarding rigidity of binding of AMs.
16. Plotting the dD values (Y axis) at different time intervals against dF values at the same interval reveals information regarding the kinetics of binding.
17. Calculate the number of molecules of AMs adsorbed onto the PS surface and/or to each other using Avogadro's formula (see Note 13).

3.5. Analysis and Graphical Representation of the Data

The data can be analyzed manually or using the software provided with the equipment, and is either graphically presented as shown in Figs. 3 and 4 using Excel/other graphing programs or tabulated as shown in Tables 2 and 3.

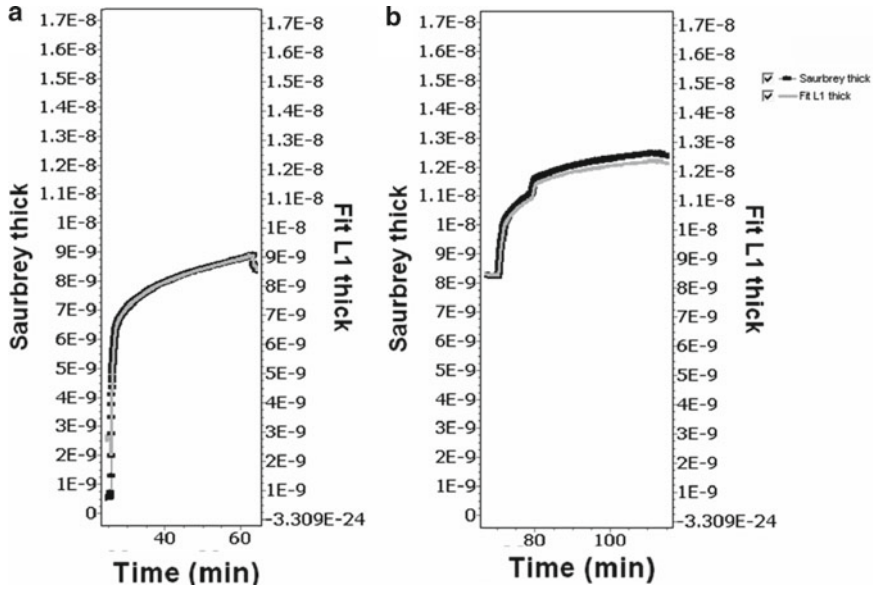

Fig. 2. Fit analysis for the adsorption of C3 onto VCP adsorbed onto PS. The values of Saurbrey thickness (*Y*1-axis) and Fit L1 thickness (*Y*2-axis) obtained after Fit analysis done using the QTools software coincide with each other indicating that the adsorption of VCP on PS (**a**) and C3 on VCP (**b**) obeys Sauerbrey's equation.

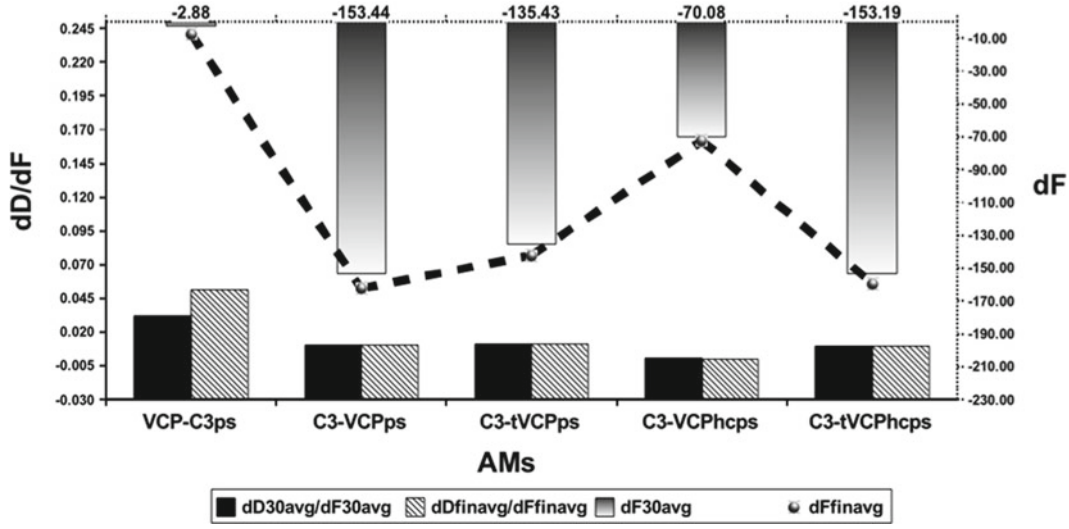

Fig. 3. d*D*/d*F* ratios (the primary *X* and *Y* axis) and d*F* values (the secondary *Y* axis [*right*] and the secondary *X* axis shown [*top*]) for binding of VCP, tVCP, and C3 on PS. d*D*/d*F* ratios at a 30-min interval are shown by *solid black bars*, whereas final d*D*/d*F* ratios are shown by *hatched bars*. The dF_{30} values are shown by the *gray gradient bars*. The dF_{fin} values are shown by a *black broken line with black dots*. The d*F* and d*D* values shown indicate the average values obtained from the two experiments unless specified in Table 1. "hc" and "ps" in "hcps" stand for high concentration and polystyrene surface, respectively. See Note 14. Figure modified from ref. 4 published in the Open Journal of Biochemistry.

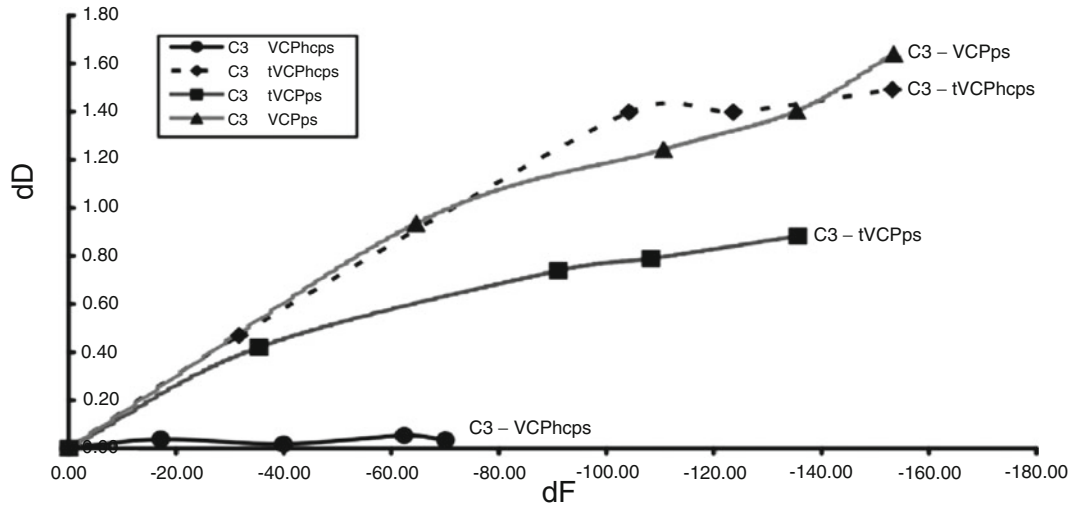

Fig. 4. Absorption of C3 on VCP and tVCP absorbed on PS crystal surface. d*F* values for the interaction of VCP and tVCP adsorbed on the PS surfaces with C3 (*X*-axis) were plotted against d*D* values (*Y*-axis) at five different time points. The data label for each series indicates AMs (Series: C3-VCPhcps, C3-tVCPhcps, C3-tVCPps, and C3-VCPg indicate adsorption of C3 on VCPhcps, tVCPhcps, tVCPps, and VCPg, respectively. "hc" prior to ps in the case of VCP denotes VCP at high concentration) (see Notes 14 and 15). Figure modified from ref. 4 published in the Open Journal of Biochemistry.

Table 2
The number of molecules of AMs adsorbed for interaction of VCP and tVCP with C3 on PS

Sr	AMs	Final dD/dF ratio	Final dF	dF corrected	Mass (ng/cm^2)	No. of molecules adsorbed (×1,013)
1.	VCP-C3ps	0.0517	−7.3670	−5.1569	30.4257	0.6362
2.	VCPps	0.0141	−24.7845	−17.3492	102.3600	2.1403
3.	tVCPps	0.0137	−35.4495	−24.8147	146.4064	4.6897
4.	VCPhcps	0.0340	−140.8510	−98.5957	581.7146	12.1635
5.	tVCPhcps	0.0124	−35.9400	−25.1580	148.4322	4.7546
6.	C3ps	0.0105	−177.9875	−124.5913	735.0884	2.3298
7.	C3-VCPps	0.0099	−162.2495	−113.5747	670.0904	2.1238
8.	C3-tVCPps	0.0110	−142.2435	−99.5705	587.4657	1.8620
9.	C3-VCPhcps	0.0002	−72.4925	−50.7448	299.3940	0.9489
10.	C3-tVCPhcps	0.0094	−159.2420	−111.4694	657.6695	2.0845

Sr serial number, *AM* adsorbing moiety, *PS* polystyrene, hc in "hcps" stands for high concentration (see Note 14), and d*F* corrected refers to the d*F* values corrected for water bound to the protein molecules (see Notes 13, 16, and 17)

Table 3
The ratio of binding of the number of molecules of AM1 to AM2

Sr	Binding moieties (AM1:AM2)	Ratio AM1:AM2
1.	VCPps:C3-VCPps	12.818:1
2.	tVCPps:C3-tVCPps	2.519:1
3.	C3ps:VCP-C3ps	3.662:1

3.5.1. Nature of Binding

To study the viscoelastic properties of binding (rigidity or strongness of binding), the dD/dF ratio at a particular time point (30 min and/or final values) and dF values at the same time point can be plotted (Fig. 3). In addition to the viscoelastic properties of interaction of compounds with the complement component C3, the dF values give information regarding the amount of AMs adsorbed at that time point.

Low dF values indicate no or little adsorption (e.g., VCP-C3ps in Fig. 3). dD/dF ratio indicates rigidity of binding. As shown in Fig. 3, the order for dD/dF ratios for the different AMs is (C3 vs. VCPhcps) Ð (C3 vs. VCPps) = (C3 vs. tVCPps) = (C3 vs. tVCPhcps) < (VCP vs. C3ps). The ratio was the lowest for C3 vs. VCPhcps with dD/dF ratio approaching zero, suggesting very strong binding (see Note 14).

3.5.2. Kinetics of Binding

Figure 4 shows the kinetics of binding of AMs. In this graph, the dD values (Y-axis) at five different time points (0, 1, 5, 15, and 30 min) are plotted against dF values (X-axis) at the same time points (see Note 15). The dD values change linearly with dF values during the first phase of adsorption (i.e., the adsorption of C3 on PS, or on VCP and tVCP). In most other cases, this first phase is followed by the second phase, where dD values either increase or decrease with the change in dF values. In some cases, the adsorption of AMs may constitute a single phase (e.g., the adsorption of C3 on VCPhcps as shown in Fig. 4). A single-phase adsorption may be due to formation of a monolayer of the AM and the second phase may indicate the formation of a bilayer.

3.5.3. Number of Molecules of AMs Adsorbed onto the Surface and/or on the Other AM

Calculation of the mass of the AMs adsorbed onto the crystal surface or on the other AM using dF values is shown in Table 2. Calculate the number of molecules of the AMs adsorbed onto the surface of the crystal or onto the other AM using the Avogadro's formula and mass adsorbed (see Notes 13 and 16–18). Calculation of the ratio of the AMs adsorbed onto the surface or the other AMs as shown in Table 3.

4. Notes

1. UV chamber generates UV light at the specific wavelengths of 185 and 254 nm, and is used to remove organic contaminants on the crystal surface.
2. As examples for this chapter, we show data with VCP and complement component C3. Purified VCP and an N-terminal truncation of VCP (tVCP) expressed in *Pichia pastoris* yeast expression system, were purified by using a Hi-trap heparin column. tVCP lacks the first short consensus repeat (SCR) domain of VCP and, thus, is a histidine-tagged protein comprising the 2nd, 3rd, and 4th SCR domains of VCP. In the work described in this chapter, the final concentration of tVCP was 1.8767 times higher than that of VCP supplied and its molecular weight (18.8 kDa) is 1.5319 times less than that of VCP (28.8 kDa). Thus, in order to prepare tVCP at the same concentration of VCP, 40.82 to 76.06 μl of tVCP was dissolved in 2 ml of PBS solution prior to experimentation. To prepare C3, 15 μl of 2 mg/ml C3 was diluted in 2 ml PBS prior to the experiment. C3 was kindly provided by Prof. Krishna Murthy's laboratory, University of Alabama, Birmingham, AL, USA.
3. The crystals can also be cleaned using 1:1:5 parts of ammoniac:H_2O_2(30%):H_2O. Water used in the experiment and/or to wash the sensor crystal surface was of deionized double-distilled Millipore grade, and it was degassed using nitrogen gas prior to use.
4. When the resonant frequencies of the sensor crystal do not match the values provided in the instruction manual, the sensor crystal needs to be discarded. However, sometimes the resonant frequencies may not match the values provided in the manual due to inadequate washing and drying steps. So before discarding, first try to rewash the sensor crystal and the chamber. Then, dry them with the nitrogen gas, subject it to the UV light as mentioned in the protocol, and check the resonant frequencies again.
5. For all the experiments, only the crystals showing comparable and similar pattern of f and D values were used.
6. In most of the Q-sense-based experiments, temperature needs to be controlled appropriately by use of the controller knob on the electronic unit. Experiments can be carried out at various temperatures between 15 and 40°C. Extremes of temperature may induce aberrant changes in frequency. For the protocol described here, the experiments were carried out at 25°C. Before adding the solution onto the sensor crystal, make sure

that the temperature of the solutions of all the AMs is brought to the room temperature and is nearly the same.

7. The position of the sample holder (Fig. 1c) can be adjusted to optimize the flow rate. The same flow rate should be maintained in all the experiments.
8. It is recommended that the final volume of each AM to be used should be about 2 ml for an accurate measurement.
9. To prepare VCP solution, approximately 50 μl of 1.2 mg/ml of endotoxin-free purified VCP was diluted to 2 ml using ice-cold PBS solution at the time of experiment. The tVCP used in the study was purified by desalting and by washing with PBS and concentrated by passing the solution several times through a 5-kDa cutoff filter.
10. If the adsorption does not follow Sauerbrey's equation, a method called Z-match and/or viscoelastic modeling is recommended. All the instructions regarding modeling are provided with the software.
11. Orientation of AMs may be important for binding. For example, we found that VCP does not bind to C3 when C3 is first adsorbed onto to the crystal surface. Under such circumstances, before making any conclusion, the sequence of addition of AMs should be reversed to check the binding orientation.
12. The surface of the sensor crystal may also be coated with a monolayer of specific antigen, antibody, or any other protein/peptide under investigation. Many types of sensor crystals are also available for specific use. For example, PEG-COOH and/or amine coupling reactions can be used with certain surfaces.
13. If the water binding capacity of AMs is not known, the number of molecules bound to each other and with the surface will not be a true reflection, but an approximate value. In order to get exact values of the number of molecules of AMs binding to each other or to the sensor crystal surface, the water bound to proteins should be determined experimentally. Optical techniques, such as ellipsometry (ELM) or optical wave guidance lightweight microscopy (OWLS), give information regarding the "dry mass" of the protein adsorbed. QCM-D gives information regarding "wet mass (Protein plus water bound)." So the information regarding water bound to the protein adsorbed could be calculated by combining the optical techniques with QCM-D as described by Höök et al. (32). Alternatively, one can estimate that approximately 30% of the mass of protein molecules accounts for the water bound to them, and therefore for the current example, it was deducted from the final mass of AMs interacting with the surfaces or other AMs.

14. VCPhc means VCP at higher concentration. The concentrations of VCPhc and tVCPhc were 2.5 times higher than those of VCP and tVCP, respectively.
15. The zero values used in the dD vs dF graphs as shown in Fig. 4 do not indicate a time point or a data point, but only the starting point of the graph. These graphs are plotted to avoid time dependency and reveal the shapes of the graph in order to study the phases of adsorption of AMs either on the surface or on the other AMs.
16. For calculating the number of molecules of AMs adsorbed onto the surface of the crystal or onto the other AM using Avogadro's number, the molecular weights of the AMs must be expressed in nanograms because the mass of the AMs adsorbed is expressed in the same unit. For the current example, the molecular weights of C3, tVCP, and VCP in nanograms (ng) were $1{,}900 \times 10^{11}$, 188×10^{11}, and 288×10^{11}, respectively. These values were taken either from the Calbiochem catalog (C3) or from the literature (VCP and tVCP).
17. When the number of molecules of water bound to the AM is not known, approximate values of water of hydration of the protein molecules based on literature values are used to calculate the mass deposited using Sauerbrey's equation, and the number of molecules adsorbed on the surface using Avogadro's equation.
18. Although not shown here, to get more information regarding the viscoelastic properties of the AMs, modeling with QTools software provided with the equipment is an option.

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