Development of SPR Methods to Evaluate Peptide Inhibition of Cadherin Interactions

By

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ABSTRACT

The blood-brain barrier (BBB) is formed by vascular endothelial cells that selectively restricts the entry of molecules into the brain. Due to this selectivity, a lot of drug molecules are obstructed from entering the brain. Therefore, overcoming the BBB has been proven challenging in the field of medicine. Cadherin proteins (i.e., E- and VE-cadherins) are cell-cell adhesion molecules in the BBB that play a very important role in maintaining the *adherens* junction. Our group has shown that the BBB intercellular junctions can temporarily be disrupted by synthetic cadherin peptides (i.e. HAV6 and ADTC5) to improve passive penetration of molecules through the paracellular pathways of the BBB. Cyclic ADTC5 peptide binds to cadherins to block cadherin-cadherin interactions in an equilibrium and reversible fashion at the intercellular junctions to increase the porosity of paracellular pathways of the BBB. ADTC5 peptide has been used to deliver large molecules such as lysozyme, albumin, and monoclonal antibody (mAb) into the brains of animal models. This study develops Surface Plasmon Resonance (SPR) methods to confirm (a) interaction of EC1-to-EC1 domain of cadherins; (b) binding of ADTC5 peptide EC1 domain of cadherins; and (c) inhibition EC1-to-EC1 interaction by ADTC5. Our results showed that the EC1 domain of E-cadherin in the solution phase can bind to EC1 domain of E-cadherin immobilized on the surface of carboxylic acid SPR chip (KD of 1.45 μM in TBS and 44 μM in PBS). In addition, the EC1 domain of VE-cadherin in solution phase binds to the EC1 domain of E-cadherin immobilized on the surface of SPR chips (KD of 10.15 μM in TBS using gold chip; 47.7 μM in TBS and 1,340 μM in PBS using carboxylic acid chip). ADTC5 inhibits the EC1-to-EC1 interactions for E-cadherin with IC_{50} of 4.27 μM and the EC1-to-EC1 interactions between E- and VE-cadherins with IC_{50} of 3.27 μM. In the future, this method could be used to evaluate inhibitory activities of new cadherin peptides designed using computational chemistry.

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CHAPTER 1. INTRODUCTION

1.1. The Blood-Brain Barrier and Its Components

The blood-brain barrier (BBB) is a highly selective endothelial cell barrier which separates the blood from the brain and extracellular fluid in the central nervous system (CNS).[1-3] It consists of over 100 billion capillaries, and they are formed by endothelial cells of the capillary wall and astrocyte end-feet which are ensheathed by pericytes embedded in the capillary basement membrane.[3-6] The BBB is set up in such a way to allow the passage of some selective molecules by passive diffusion and transporter-mediated uptake of specific molecules such as glucose, water and amino acids which are crucial to neuronal function. Thus, the blood-brain barrier acts effectively to protect the brain from unwanted molecules and pathogens.[1, 7]

The BBB is regulated by calcium-dependent and calcium-independent proteins called cadherins at the *adherens* junction while the desmocollins and desmogleins regulate the desmosomes.[8, 9] Cadherins are calcium-dependent transmembrane proteins while Nectins are calcium-independent proteins; they hold and connect the cells together by mediating cell-cell adhesion. Cadherin proteins from the opposite cells form homophilic *trans*-interactions as a "Velcro" to glue the cells of the BBB together to make capillaries (**Figure 1**).[10-13]

This work is focused on (a) developing SPR methods to evaluate the characteristics of EC1-to-EC1 domain interactions (i.e., E- and VE-cadherins), (b) confirming the binding of cadherin peptides (i.e., ADTC5) to the EC1 domain of E-cadherin, and (c) developing a peptide inhibition assay for cadherin-cadherin interactions. Both of E- and VE-cadherins are believed to be present in the intercellular junctions and they are in the classical cadherin family with five Extra Cellular (EC) domains (i.e., EC1–EC5 domains). In both types, the EC1 domains are involved in *cis* or *trans* cadherin-cadherin interactions. The *cis*-interactions occur between the EC1 domain of one

cadherin to the EC2 domain of a neighboring cadherin from the same cell surface (**Figure 1A**). The *trans*-interactions are created by binding between the EC1 domain of cadherin from one cell membranes to the EC1 domain of another cadherin form the membranes of opposing cells (**Figure 1A**). The formation *trans*-interaction is facilitated by the "β-strand-swap" between the two N-terminus of opposing EC1 domains. The Trp residue from one EC1 domain is docked into hydrophobic pocket of the other EC1 domain, which is called "domain swapping" (**Figure 1B**).[14, 15]

E-cadherin is a classical cadherin which falls under Type I cadherin that forms bonds with

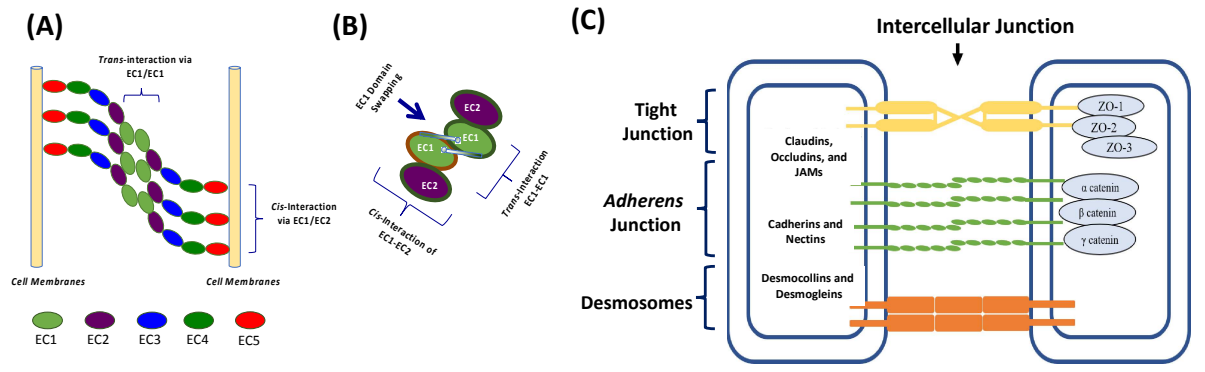

Figure 1. (A) The proposed *cis*- and *trans*-interactions between cadherins. The *cis*-interactions are facilitated by the EC1-to-EC2 interaction between two cadherins from the same cell membranes. The *trans*-interactions are mediated by the EC1-to-EC1 interaction between two cadherins from the opposing cell membranes. **(B)** A model for the "domain swapping" interaction between two EC1 domains in *trans*-interaction. **(C)** The structure of the intercellular junction of the BBB with protein-protein interactions at tight junction, *adherens* junction, and desmosome.

itself and other Type I cadherin molecules such as N-cadherin and P-cadherin. VE-cadherin on the other hand is a Type II cadherin, and primarily forms homophilic bonds.[16] The primary difference in Type I and Type II cadherin proteins is in the mechanism of domain swapping. Type II cadherins

have two conserved Trp residues on their EC1 domains, as opposed to one Trp residue in case of Type I cadherins. This makes the strand acceptor pocket in Type II cadherins larger than that of Type I cadherin proteins. As a result, it makes the homophilic interaction between Type II cadherins stronger than homophilic interactions of Type I cadherins.[13, 17, 18]

It is predicted that Type I and Type II cadherins would not form bonds between themselves, but VE-cadherin is an anomaly in the family of Type II cadherin. It has many features which are similar to Type I cadherin proteins. Although the EC1 domain of VE-cadherin has two tryptophan residues, in accordance with Type II cadherin proteins, the surface interface for the strand exchange is more similar to the Type I cadherins.[19] In addition, the surface interface of VE-cadherin resembles that of Type I cadherin proteins. This leads to the possibility that VE-cadherin may interact with Type I cadherin, more specifically E-cadherin because the possibility of their presence on the BBB. This study explores the EC1-to-EC1 interaction of between E-cadherins as well as the EC1-to-EC1 interaction between E- and VE-cadherins.

1.2. Diffusion Through the Blood-Brain Barrier

The high selectivity of the BBB means that it blocks almost all pathogens and unwanted molecules from the blood stream to enter the Central Nervous Systems (CNS), but it also means that the BBB itself impedes the delivery of drug molecules into the brain. Therefore, limited permeability of the BBB hinders development of effective treatments from various brain diseases such as brain tumors, multiple sclerosis (MS), Parkinson's, and Alzheimer's disease (AD). Because of this, it is believed that around 98% of available drugs cannot be used for CNS diseases.[20, 21]

Lipinski's Rule of Five was developed to predict drug candidates that can passively cross the intestinal mucosa barrier. The rule was developed based on correlation between observed transport properties of drug molecules and their physicochemical properties. The study also describes the important molecular properties relevant to pharmacokinetics (PK) characteristics such Absorption, Distribution, Metabolism and Excretion (ADME).[22, 23] Lipinski's Rule of Five states that the candidate drug molecule:

i) should not have more than 5 hydrogen bond donors (total number of N-H and O-H bonds);
ii) should have no more than 10 hydrogen bond acceptors (all O and N atoms);
iii) should have molecular mass less than 500 Daltons;
iv) should have an octanol-water partition coefficient (Log P) that does not exceed 5.

Lipinski's Rule of Five extends to the drug penetration through CNS, as the drug activity in the CNS is governed by a Simple Rule of Two:[24, 25]

i) if the number of nitrogen and oxygen atoms in a molecule is less than or equal to five, it is likely to penetrate through the BBB and enter the brain and
ii) if logP(N + O) is positive then the compound can enter CNS.

To improve delivery of drugs into the brain, it is necessary to understand the mechanism of transport of molecules through the BBB to optimize their efficacy. The transcellular and paracellular pathways are two mechanisms by which molecules permeates through the BBB. In transcellular pathway, molecules are transported through the cell; the main modes of transport of molecules are via passive diffusion and receptor-mediated transport. In passive diffusion, the movement of molecules from the blood to the brain is defined by the concentration gradient, where

molecules move from a region of high concentration in the blood to a region of low concentration in the brain. In paracellular transport, the molecules passively diffuse through the intercellular junction between the cells and this passive permeation is limited by the tight junctions. The tight junctions that are mediated by occludins, claudins, and junctional adhesion molecules (JAMs) to limit molecules to passively cross via the paracellular pathway (**Figure 1C**).

The *adherens* junction that resides below the tight junction and is connected by cadherins and nectins to maintain the cell-cell adhesion in a highly dynamic and reversible manner. Therefore, these characteristics open a new way to explore modulation of cadherin interactions using cadherin peptides to create larger pores in the intercellular junction to increase passive diffusion of molecules from the blood through the BBB paracellular pathway into the brain.

Our laboratory has exploited the paracellular pathway to improve drug transport across the BBB with the help of various cadherin peptides that disrupt cadherin-cadherin interactions. Cadherin peptides (i.e., HAV6 and ADTC5) have been shown to increase *in vivo* brain delivery of ^{14}C-mannitol,[26] ^{3}H-daunomycin,[26] adenanthin,[27] gadopentetic acid (Gd-DTPA),[1, 28, 29] IRdye R800,[28] peptides,[30] and proteins (i.e., lysozyme, albumin, monoclonal antibody).[30-32] Other researchers have also evaluated tight junction modulators such as EDTA, EGTA, and citrate to improve paracellular permeation of molecules across MDCK and Caco-2 cell monolayers.[7] Palmitoyl carnitine, deoxy- or glycol-cholate, chitosan, and hypertonic mannitol have also been found to increase transport of molecules through the BBB.[7, 33] The majority of these methods to enhance paracellular passive diffusion were through non-specific disruptions of the intercellular junctions. In contrast, cadherin peptides modulate the intercellular junctions by specifically inhibiting of cadherin-cadherin interactions.

It was previously believed that drug molecules for treatment of diseases such as Multiple Sclerosis (MS), Alzheimer's Disease (AD), and brain tumors cannot readily permeate the BBB, so these diseases have been difficult to treat. Recently, our laboratory utilized ADTC5 peptide to deliver brain-derived neurotrophic factor (BDNF) into the brains of experimental autoimmune encephalomyelitis (EAE) mice, an animal model for MS.[34] The results showed that the EAE mice treated with a mixture of BDNF and ADTC5 did not show any disease relapse after remission while the EAE mice treated with BDNF alone and vehicle showed disease relapse.[34] The BDNF +ADTC5 group also had improved remyelination and neuroregeneration. Treatments with a mixture of BDNF and ADTC5 in an animal model for Alzheimer's disease (*i.e.*, double transgenic APP/PS1 mice) improved cognitive behaviors of the animals in Y-maze and novel object recognition (NOR) assessments as well as induced neuroregeneration.[35] These results indicate that ADTC5 can enhance brain delivery of BDNF.

Because of the *in vivo* activity of ADTC5, it is important to get a better understanding of the mechanism of action of cadherin peptides (*e.g.*, ADTC5) to inhibit cadherin-cadherin interactions and to develop a rapid method to evaluate new and selective derivatives as inhibitors of cadherin-cadherin interactions. Therefore, this study explores the binding properties between the EC1 domains of E- and VE-cadherin using Surface Plasmon Resonance (SPR), to get an estimate of the binding constants of affinity between two EC1 domains of cadherin molecules. The second part of the study involves inhibition studies of EC1-to-EC1 interactions by ADTC5.

1.3. Surface Plasmon Resonance to Study Protein-Protein Interactions

SPR has been used to understand quantitative properties of molecular interactions such as large biomolecules like proteins and glycans. One of the molecules, the ligand, is immobilized on a thin film surface. The other molecule, the analyte, is kept in a mobile phase. Injecting the analyte over

the ligand produces a change in the Refractive Index, known as a signal which is quantified in a precise manner. The method relies on the change of molecular mass during binding of the analyte to the immobilized ligand; therefor, there is no need to label the interacting components, thereby eliminating the changes of their molecular properties.[36] Because there is no need to label the molecules, SPR presents a great advantage over other methods and it minimizes the complexity of binding studies. SPR assays also provide real time information of interaction between ligand and analyte molecules within a broad range of concentrations. Finally, this assay requires only small sample volumes (around 200 μL) and can provide accurate data with low purity of one of the reactants.[37, 38]

SPR assays have been successfully used to study cadherin interactions by various researchers and they minimize the time for determining the essential biding parameters.[39] Inhibition of protein-protein interaction monitored by SPR has been used to obtain the inhibitor IC_{50} value.[40, 41] Binding affinities between the proteins (KD) are calculated from the ratio of the rate constants (K_{on} and K_{off}), which in turn are determined from the nature of the curves produced when the proteins interact with each other. Therefore, we hope to exploit the principles of SPR in its full potential to determine binding and inhibition of the EC1-to-EC1 of E- and VE-cadherins.

CHAPTER 2. EXPERIMENTAL PROCEDURES

2.1. Transformation of Plasmids into *E. Coli*

A similar protocol was carried out to express both EC1 domains of E- and VE-cadherins. The c-DNA sequences of E- and VE-cadherin proteins from human epithelial and endothelial cells were inserted into the pASK-IBA6 plasmid. The plasmid carries the AmpR gene, f1 origin, Multi Cloning Sites (MCS) which consists of the Streptag-1, Factor Xa, and EC1 gene. The Streptag-1

sequence is “WSHPQFEK” and it is essential in purification of the protein by affinity chromatography using StrepTactin column. The sequences of the EC1 domains of the two proteins are given in Table 1.

Table 1. Sequences of the EC1 Domains of E- and VE-Cadherin Proteins

EC1 Domain of E-Cadherin	EC1 Domain of VE-Cadherin
WSHPQFEKIEGRDWVIPPISCPENEKGPFPKN LVQIKSNKDKEGKVFYSITGQGADTPPVGVFI IERETGWLKVTEPLDRERIATYTFSHAVSSNG NAVEDPMEILITVTDQNDNKPEFTQEVFKGS VMEGALPGTSVMEVTATDADDD	WSHPQFEKDWIWNQMHIDEEKNTSLPHHV GKIKSSVSRKNAKYLLKGEYVGKVFRVDA ETGDVFAIERLDRENISEYHLTAVIVDKDTG ENLETPSSFTIKVHDVNDNWPVFTHRLFNA SVPESSAVGTSVISVTAVDADDP

To start the expression process, the plasmids containing the desired sequences were retrieved from the refrigerator (–20°C) and the bacterial cells (*E. Coli* BL21) were retrieved from the ultra-low freezer (–80°C). They were allowed to thaw under ice for 15–20 minutes. After the thawing process completed, 2–5 µL of plasmid DNA was mixed with 50 µL of bacterial cells in a sterile Eppendorf tube using pipette tip.

The vial containing the mixture was kept under ice for 30 minutes, and immediately after that, it was put in water bath at 45°C, and then followed by putting it in ice again for 5 minutes. This step is known as the heat shock method. A sudden increase in temperature creates pores in the plasma membrane of the bacteria and allows for plasmid DNA to enter the bacterial cell.

SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) was prepared beforehand and was kept ready at 37°C. The

mixture in the vial was then transferred to 200 µL of SOC medium. SOC medium is normally used in the recovery phase of cells because it contains glucose.[42] The mixture in SOC medium was incubated in the shaker at 250 rpm for 1 h at 37°C.

LB media containing 5 g peptone, 5 g NaCl, 2.5 g Yeast Extract, 10 g agar in 500 mL was prepared beforehand for growing the cells on plates. After the LB media was autoclaved, 500 µL Sodium-Ampicillin salt solution was added. Then, 50 µL and 150 µL of transformed cells in SOC medium were spread on 2 separate plates containing LB media. It is recommended to spread different amounts of cells on the culture plates to find a good condition for generating optimal bacterial colonies. This also helps the growth of clear colonies across the plate. The plates were incubated for 14–16 h at 37°C. After incubation, colonies of bacteria were observed on the plates with cells of interest that survived in the media containing ampicillin. The role of ampicillin was to kill off all the bacterial cells that were not carrying the ampicillin resistant (AmpR) gene in their plasmid. As a result, only our cells of interest would survive in the media, since they have the AmpR gene in their plasmid.

2.2. Expression of the EC1 domains of E- and VE-cadherins

Initially, LB broth (2 L containing 10 g Peptone, 10 g NaCl, and 5 g Yeast Extract in 500 mL) was prepared and it was divided into 4 flasks with each containing 500 mL of broth. Then, the flasks containing the broth were autoclaved. From each of the flasks, 10 mL of broth was taken out and kept aside in smaller flasks. In each of the small flasks containing 10 mL of media, one single clear colony from the plates was added carefully using a pipette tip. This was done to avoid substrate inhibition. The sterile conditions were maintained to prevent unwanted growth of

unwanted bacteria in the broth. The flasks were then set for incubation for 12 h at 250 rpm and 37°C. Airtight conditions were maintained to prevent growth of unwanted bacteria in the medium.

On the next day, the media became turbid, signifying bacterial growth. Then, the media from the small flasks with 10 mL cell solution were then transferred to the bigger flasks containing 490 mL media followed by incubation for 2 h. After 2 h, the optical densities (ODs) of the cultures were checked at 550 nm until they have reached 0.6 to indicate that the cells have reached the exponential phase of the cycle and were ready for the induction.

Anhydrotetracycline (30 μL) was added into each culture flask as an inducer and the cultures were further set for incubation for 6 h. After 6 h, the cultures were centrifuged several times at 4500 rpm for 20 min to produce dense pellets. The supernatants were discarded and the cell pellets were stored in falcon tubes at –80°C for a later isolation and purification process of the EC1 proteins.[43] The cells were cryo-preserved using 50% glycerol and then stored in the ultra-low temperature freezer to eliminate the future need for the process of bacterial transformation for the EC1 domains of E- and VE-cadherin.[44]

2.3. Purification of the EC1 Domains of E- and VE-cadherins

The falcon tubes containing the cells were taken out from the –80°C freezer and the frozen cells were suspended in 20–25 mL of running buffer (100 mL Tris HCl, 150 mM NaCl, 1.0 mM EDTA, 0.02% w/v NaN_3) for 30 minutes to slowly thaw the cells. The cells were then vortexed until the solution was homogenous. Then, the cells were lysed with a French Press machine and the process was repeated for six times for complete cell lysing. The completed lysis process can be affirmed by a change in solution color to become a tint of yellow color. The resulting solution was then centrifuged at10,000 rpm and 4°C for 1 h. The pellet was discarded as it contains all the

cell debris. The supernatant was collected and stored for further purification process using affinity chromatography and size exclusion chromatography (SEC).

The cell lysates for both EC1 domains from E- and VE-cadherins were subject to purification using the StrepTactin affinity chromatography column (GE Healthcare). This column contains streptavidin that recognizes the "WSHPQFEK" sequence as the Tag sequence on the EC1 domain. The EC1 domain was retained in the column due to the binding of the Tag sequence to streptavidin while remaining non-Tag proteins were eluted through the column.

Using a peristaltic pump, distilled water was purged through the column for 30 minutes set at a speed of 0.5 mL/minute. The running buffer was flowed through the column for about 5 column volumes to conditioned the column. Then, 2–3 mL of protein mixture was injected into the column followed by elution with running buffer for six column volumes to remove other proteins that were not related to the desired EC1 domain. To make sure that any of the EC1 domain was not lost, the eluates from the column were collected and analyzed using SDS-PAGE to determine the existence of the EC1 domain.

After washing, the column was treated with the elution buffer (100 mM Tris HCl, 150 mM NaCl, 1.0 mM EDTA, 0.02% w/v NaN_3, 2.5 mM DTT, 5.0 mM Desthiobiotin in 100 mL) for six column volumes to release the EC1 domain protein from streptavidin on the column. The eluted solution was collected and concentrated by centrifugation using AMICON Ultra 10 kDa molecular weight cut-off falcon tubes. Because binding affinity of desthiobiotin to streptavidin is stronger than that of the Tag sequence, desthiobiotin expels the protein from the streptavidin column.[45] The pure protein was analyzed using SDS-PAGE gel and mass spectrometry. The proteins were then stored in the refrigerator to be used later for the SPR Assays.

Because the Strep-Tactin column did not completely provide a single band of the EC1 domain of VE-cadherin, the EC1 domain from affinity column was subjected to SEC in AKTA Pure FPLC (GE Healthcare, Inc.) in phosphate buffer at pH 8.0. Prior to protein sample injection, the SEC column was primed with the buffer for five column volumes. Then, the protein sample (5 mL) was injected into 150 mL Superloop for each run. The protein was eluted in an isocratic condition with five column volumes. The sample eluted out of the column was collected at 5 mL per tube using a fraction collector. Finally, the column was washed with 20% ethanol for two column volumes to condition it for future use.

To check whether the method was working properly, a test run was done using bovine serum Albumin (BSA, 65 kDa), Ovalbumin (45 kDa), and Lysozyme (14.5 kDa). These proteins were mixed together at 1.0 mL each (0.01 mM) followed by dilution to 5 mL with pure water. The chromatogram showed three distinct peaks, signifying elution of the three proteins. The eluted proteins were also analyzed with gel electrophoresis to make sure they were congruent with the expected elution form SEC in which the largest protein would be eluted first.

2.4. Quantification of the EC1 Domains

BCA assay was used to quantify the amount of protein in solution. In this case, peptide backbone of the protein reduces Cu^{2+} to Cu^{1+} in an alkaline solution to produce a purple color, that can be detected at 562 nm, due to chelation of Cu^{1+} by two bicinchoninic acids. The intensity of the purple color corresponds to concentration of the protein in solution. Thus, a standard curve is generated using different concentrations of BSA by determining the absorbance at 562 nm for each

concentration. The EC1 domain concentration is determined after interpolating of its absorption into the standard curve.

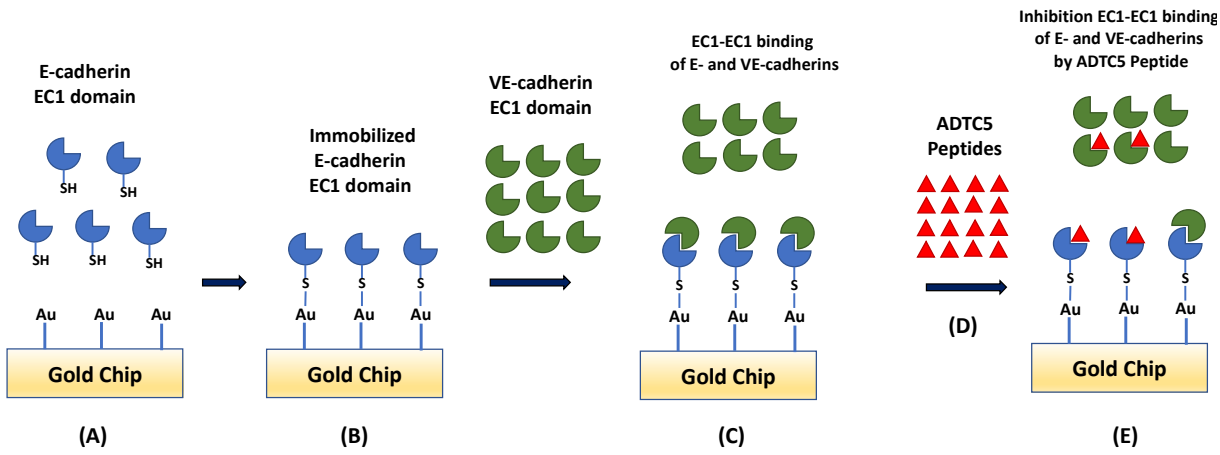

Figure 2. The development of SPR assay using gold chips to study binding between the EC1 domain of E-cadherin and the EC1 domain of VE-cadherin as well as inhibition of the binding using ADTC5 peptide. **(A)** Reaction of a thiol group on the Cys residue of E-cadherin EC1 domain to Au atoms on the surface of the gold chip to give **(B)** the immobilized E-cadherin EC1 domain via the S-Au covalent bond. **(C)** The VE-cadherin EC1 domain was eluted through the chip to determine the KD of the EC1-to-EC1 binding between E- and VE-cadherins. **(D)** Addition of ADTC5 peptide to solution of the VE-cadherin EC1 domain was done to inhibit EC1-to-EC1 binding between E- and VE-cadherins. **(E)** Binding of ADTC5 to the EC1 domains of E- and VE-cadherins inhibited the EC1-to-EC1 interaction to determine the IC_{50} of ADTC5 peptide.

2.5. Development of SPR Assays

SPR assays were carried out using Nicoya OpenSPR and the collected data were analyzed with TraceDrawer Software. Gold and carboxylic acid chips were used in the SPR assays. The experiments were carried out in Tris buffer solution (TBS) and Phosphate buffer solution (PBS). TBS buffer contains 10 mM Tris.HCl, 150 mM NaCl, 3.0 mM $CaCl_2$, and 0.005% Tween20. PBS

buffer contains 137 mM NaCl, 2.7 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4, 10 mM $CaCl_2$, and 5.0 mM $MgCl_2$.

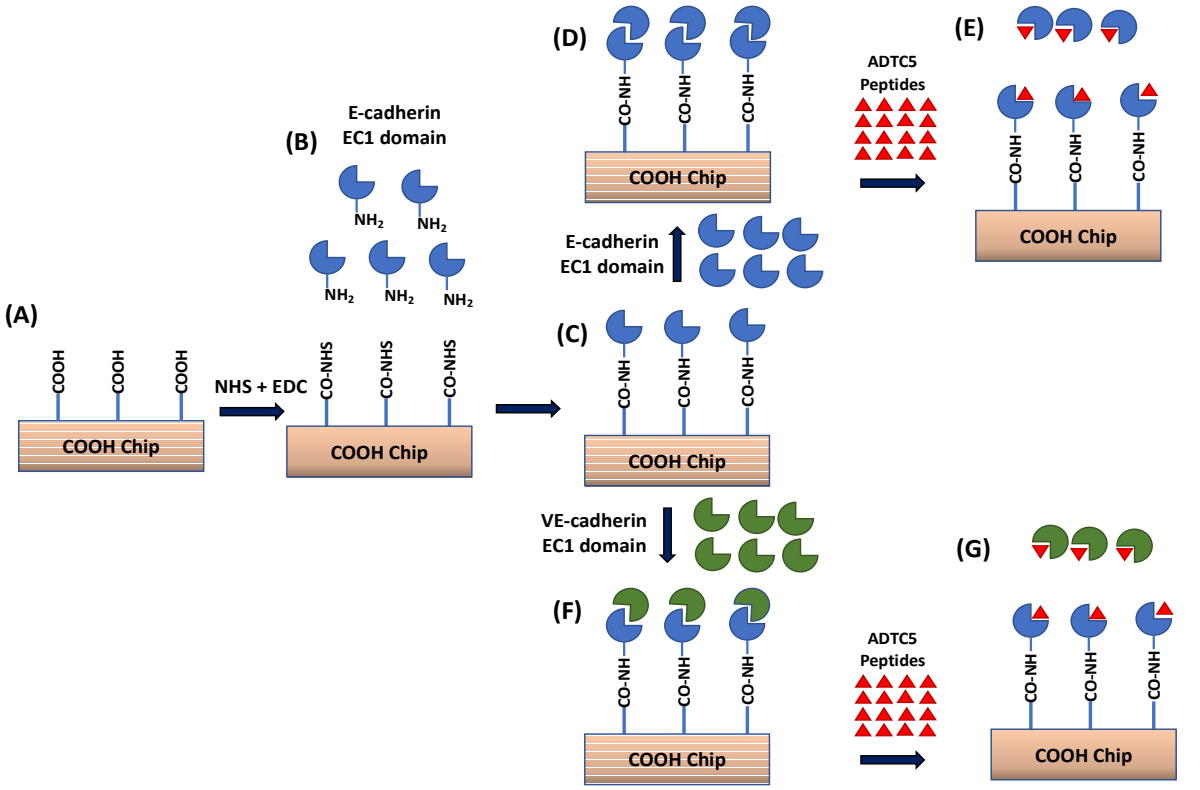

Figure 3. The SPR carboxylic acid chip was used to study binding properties of the EC1-to-EC1 domains of E-cadherins and VE-cadherin and inhibition of EC1-to-EC1 binding by ADTC5 peptide. **(A)** The carboxylic acid groups on the chip's surface were activated by EDC and NHS to produce **(B)** NHS activated esters. **(B)** The activated esters on the surface were reacted to the amino groups of N-terminus and lysine residue side chains on the E-cadherin EC1 to generate **(C)** immobilized EC1 domain of E-cadherin. **(D)** Elution of the E-cadherin EC1 domain solution through the chip's surface generated its binding to the immobilize E-cadherin EC1 domain for determining the KD of EC1-to-EC1 binding. **(E)** ADTC5 peptide was added to the solution of E-cadherin EC1 domain for inhibiting EC1-to-EC1 binding to determine IC_{50} of ADTC5 peptide. **(F)** Elution of soluble VE-cadherin EC1 domain through the immobilized E-cadherin EC1 domain was done to determine KD of EC1-to-EC1 binding. **(F)** ADTC5 peptide was added to the solution of VE-cadherin EC1 domain for inhibiting EC1-to-EC1 binding between E- and VE-cadherins to determine the IC_{50} of ADTC5 peptide.

The SPR instrument was installed with a blank chip followed for priming the system with a running buffer for 5 minutes at a flow rate of 150 μL/min. Separately, another new chip was gently washed with distilled water and air-dried. After priming, the current blank chip was taken out from the holder and a new chip was installed in its place. The new chip was subjected to running buffer at 150 μL/min until the LED detector provided a stable detector signal indicated by a flat and stable baseline. Then, 80% isopropanol was injected to remove any air bubbles in the system and a steep jump and fall signal was observed to reflect the elution of isopropanol followed by a stabilized flat baseline.

Two different ways were used to immobilize the EC1 domain of E-cadherin. First, gold chips were used to immobilized the E-cadherin EC1 domain via a Sulfur-Au covalent bond using the thiol group of the Cys residue on the protein (**Figure 2**). However, this chip cannot be used to study the EC1-to-EC1 binding of E-cadherin because the free thiol in solution E-cadherin can bind to the Au surface to give a false positive result. The immobilized E-cadherin EC1 domain can be used to study its binding to the VE-cadherin EC1 domain because it does not contain any Cys residue (**Figure 2**). Finally, the remaining open gold surface was reacted with 10% solution of poly-ethyl glycol (PEG) containing a thiol group to prevent non-specific binding of the eluted protein.

The second method is to use carboxylic acid chips to immobilized the EC1 domain of E- or VE-cadherin (**Figure 3**). The carboxylic acid groups on the chip surface were activated by injecting a mixture of 0.4 M of ethyl-(dimethylaminopropyl) carbodiimide (EDC) and 0.1 M of *N*-hydroxysuccinimide (NHS) prepared in distilled water to generate the active NHS esters (**Figure 3B**).[46] Then, 200 μL of the E-cadherin EC1 domain was injected to react amino groups of the N-terminus and Lys residue side chain to the active NHS-esters to produce peptide bonds (**Figure**

3C). It should be noted that the coupling reaction should be done immediately because the active NHS esters only stable within 3 to 5 min. The reaction between the protein and NHS-active ester produces an instant rise of the signal and injection of running buffer several times did not reduced the signal back to baseline, signifying covalent immobilization of the EC1 domain on the chip surface.

Next, the unreacted active NHS esters were capped injecting 0.1% ethanolamine solution to make amide bonds to the surface carboxylic acids. After capping, 200 μL regeneration buffer (0.1 mM HCl) is injected several times to remove unreacted reagents including the unconjugated protein on the surface. Injection of regeneration buffer showed a groove in the signal baseline followed by stabilization of the signal as a flat line, indicating that any compounds absorbed on the sensor surface have been removed. Finally, the soluble EC1 domain was injected followed by elution of running buffer. Binding of soluble EC1 to immobilized EC1 was shown as a sharp increase of the signal followed by a plateau due binding (k_{on}); then, the signal intensity drops as the bound protein released from the immobilized protein (k_{off}). After binding study, the chip was injected with regeneration buffer to remove remaining protein on the immobilized protein. The process was repeated until all different concentrations of soluble EC1 protein were injected to provide their binding signals. The binding signal data from the experiments were analyzed by TraceDrawer Software to produce KD of binding.

2.6. Inhibition of EC1-to-EC1 Binding by ADTC5 Peptide

ADTC5 (**Figure 4**) is a cyclic peptide (MW = 772 g/mol) that was synthesized and purified in our laboratory using an established procedure. The peptide was synthesized using solid phase method in Tribute Peptide Synthesizer (Gyros Protein Technologies). The cyclization was done by forming a disulfide bond (S-S) between the two cysteine residues. The peptide was purified

using semi-preparative reversed-phase C18 column on HPLC to give a purity of higher than 96% as determined using C18 analytical column on HPLC. The pure ADTC5 peptide was characterized using mass spectrometry.

To study the activity of ADTC5 peptide to inhibit EC1-to-EC1 binding, different concentrations of ADTC5 peptide was dissolved in 1 mL of distilled water. The concentration of the stock solution was 0.4786 mM and different concentrations of ADTC5 were prepared ranging

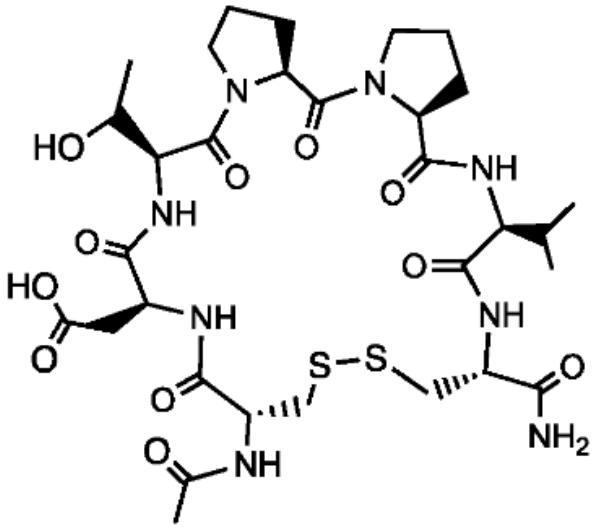

Figure 4. The chemical structure of cyclic ADTC5 peptide (Cyclo(1,7)-Ac-CDTPPV-NH_2).

from 4.79 x 10^{-5} M to 1.19 x 10^{-7} M for the homophilic EC1-to-EC1 binding of E-cadherin. For inhibition of heterophilic EC1-to-EC1 binding between E- and VE-cadherins, the concentrations of ADTC5 were ranging from 7.18 x 10^{-5} M to 2.37 x 10^{-7} M. Different concentrations of peptide were mixed with a constant concentration of the EC1 domain before injection. The competitive inhibition of EC1-to-EC1 binding by ADTC5 produces a reduced binding signal in concentration-

dependent manner (**Figure 2E and Figures 3E&G**). The IC_{50} values were obtained using MATLAB by plotting the response signals *vs.* Log concentrations of ADTC5 to produce a reverse sigmoidal plot.

CHAPTER 3. RESULTS AND DISCUSSION

3.1. Expression and Purification of the EC1 Domains of E- and VE-cadherins

The expression of the E-cadherin EC1 domain was accomplished in *E. coli* and the protein was purified using StrepTag affinity chromatography because the EC1 domain protein has a sequence that is recognize by streptavidin. For the VE-cadherin EC1 domain, the protein purification was finalized by size exclusion chromatography (SEC). The SDS-PAGE shows a single band around 20 kDa region to demonstrate a pure the E-cadherin EC1 domain (**Figure 4A**). Previously, the MALDI-TOF mass spectrometry (MS) determined that the E-cadherin EC1 domain has a molecular weight of 16.9 kDa.[47] The presence of a covalent dimer was often observed due to the formation of an intermolecular disulfide bond between two Cys residues on the EC1 domains. The MALDI-TOF also showed that the covalent dimer of the EC1 domain (MW = 33.8 kDa) formed physical dimer (MW = 66.6 kDa) and tetramer (MW = 133.2 kDa). This MALDI-TOF mass spectrometry result suggests that the EC1 domain of cadherin could form homophilic interactions with another EC1 domain as shown in the X-ray structures of C-cadherin.[13]

The VE-cadherin EC1 domain was also expressed in *E. coli* and purified using Strep-Tag column. However, the purification with Step-Tag column was not sufficient to produce a single band in SDS-PAGE gel. The SDS-PAGE showed three bands that were from monomer and oligomers of the VE-cadherin EC1 domain in reducing gel. Because the VE-cadherin EC1 domain does not have any Cys residue, these oligomers (*i.e.*, dimer and trimer) were not from a disulfide

covalent bond. Therefore, the product from the Strep-Tag column was subjected to SEC in FPLC and the SEC chromatogram showed two major peaks at 77.22 and 101.44 min. The peak at 77.22 min was from the oligomers of the VE-cadherin EC1 domain. The fractions from the peak at 101.44 min were collected and concentrated using Amicon Ultra with 10,000 kDa cut-off. The

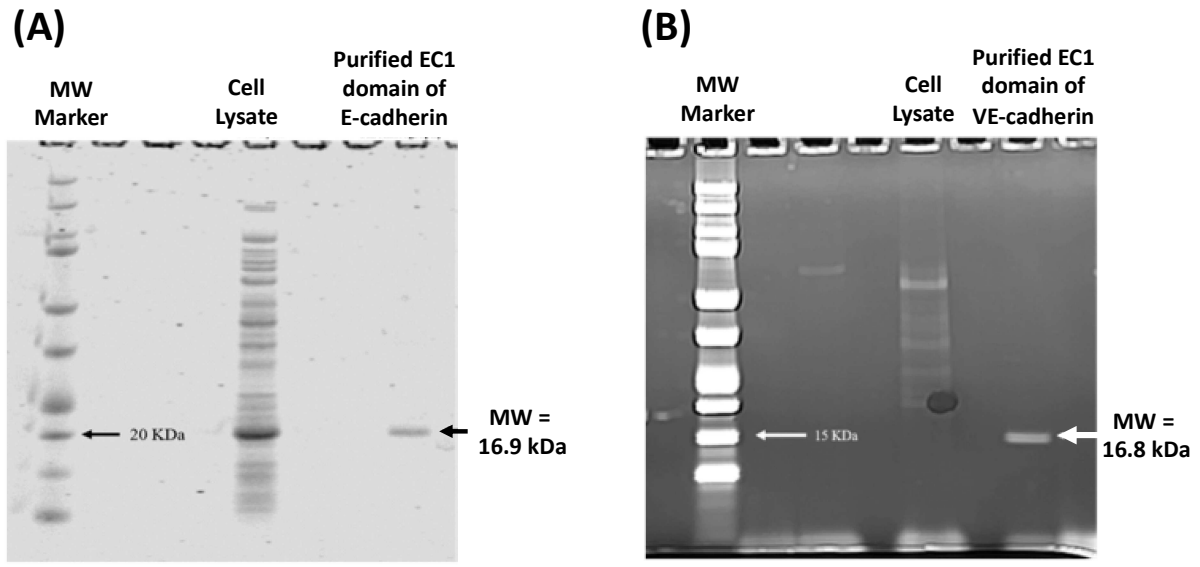

Figure 4. The results of SDS PAGE Gel Electrophoresis for the EC1 domains of **(A)** E cadherin and **(B)** VE-cadherin before and after purification. **(A)** The purity analysis of the EC1 domain of E-cadherin. The left, middle, and right lanes represent the molecular weight ladder, cell lysis, purified EC1 domain of E-cadherin, respectively. The EC1 domain of E-cadherin (MW = 16.9 kDa) was purified from the cell lysate using StrepTag affinity chromatography to give a pure single band of protein around 20 kDa molecule marker. **(B)** The purity analysis of the EC1 domain of VE-cadherin. The left, middle, and right lanes represent the molecular weight ladder, cell lysis, purified EC1 domain of VE-cadherin, respectively. The EC1 domain of VE-cadherin was purified from the cell lysate using StrepTag affinity chromatography followed by size exclusion chromatography to give a pure single band of protein with MW = 16.5 kDa. The SDS Gel electrophoresis was done using 4–12% Bis-Tris gels (ThermoFisher Scientific) and 20% MES. Coomassie Blue was used as the staining dye.

concentrated solution was analyzed using SDS-PAGE to give a single band on SDS-PAGE with a molecular weight of 16.8 kDa (**Figure 4B**).

The isolated VE-cadherin EC1 domain was also analyzed by mass spectrometry; unfortunately, the monomeric EC1 domain of VE-cadherin with MW = 16.8 kDa was not observed (**Figure 5**).

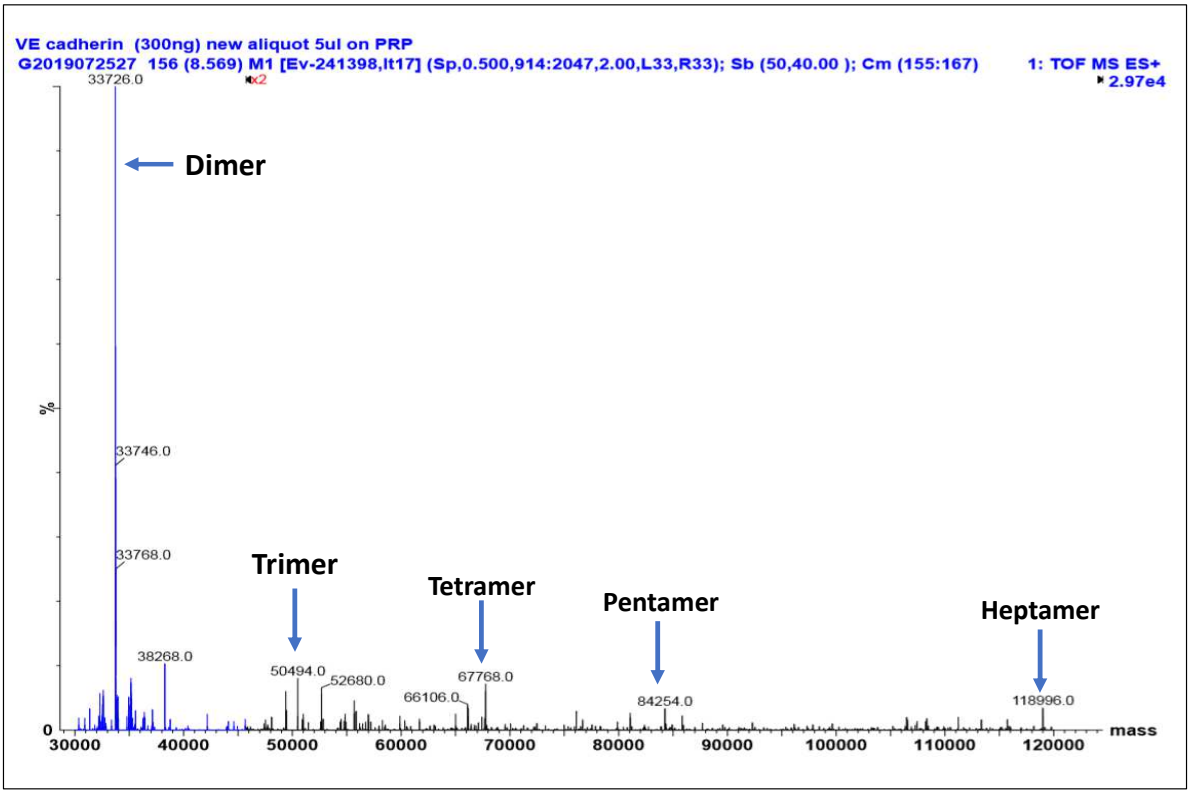

Figure 5. The mass spectrometry result of the pure VE-cadherin EC1 domain. The EC1 domain forms non-covalent or physical dimer, trimer, tetramer, pentamer, and heptamer during flight in mass spectrometry. This result suggests a tendency for the VE-cadherin EC1 domain to form homophilic oligomers.

The MS data showed dimer (MW = 33.73 kDa), trimer (MW = 50.49 kDa), tetramer (MW = 67.77 kDa), pentamer (MW = 84.25 kDa), and heptamer (MW = 118.99 kDa). These results suggest that the EC1 domain of VE-cadherin can form stable physical or non-covalent oligomers. One potential reason for stable physical oligomerization is that the EC1 domain of VE cadherin has stronger

EC1-EC1 physical binding affinity via "domain swapping". The formation of stable physical oligomers presumably due to the presence of two tryptophan residues and a large hydrophobic pocket that accepts two tryptophan residues to form homophilic binding of EC1-to-EC1 domains.[14] This also suggests that the homophilic EC1-EC1 binding of VE-cadherin as Type II cadherin is stronger than that of E-cadherin as Type I cadherin.

3.2. Evaluation of EC1-to-EC1 E-cadherin Binding using SPR

To determine the EC1-EC1 E-cadherin homophilic binding characteristic, SPR was used using carboxylic acid chip (**Figure 3**). The amino groups from the N-terminus and lysine residues were reacted to activated carboxylic acid surface to immobilize the EC1 domain of E-cadherin. The

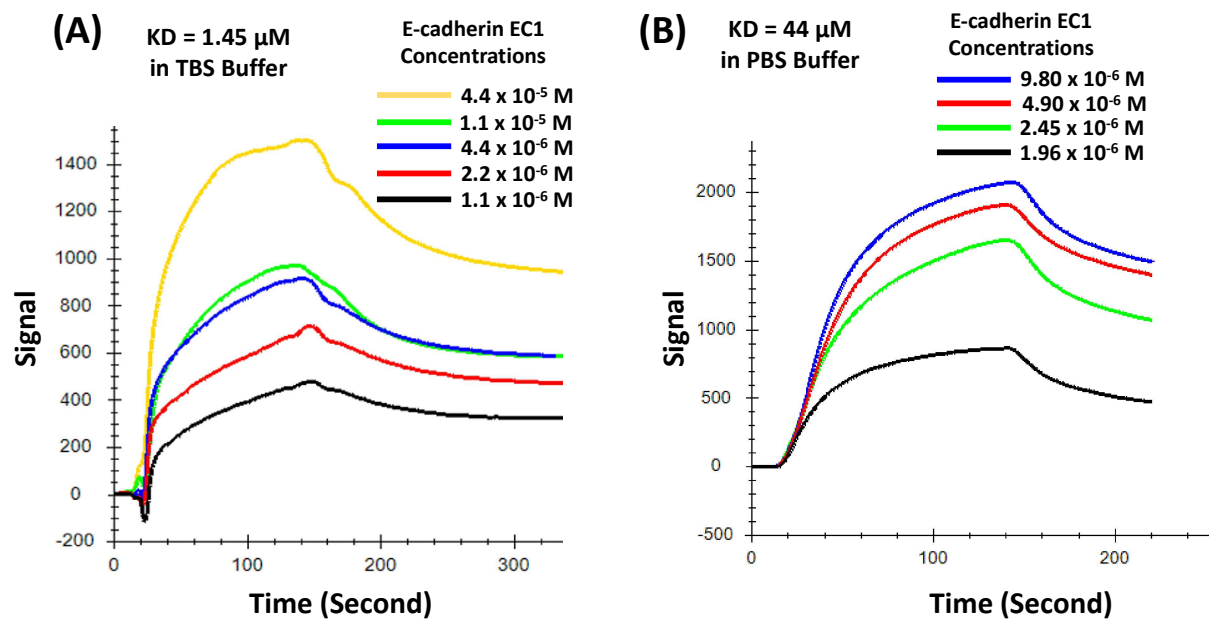

Figure 6. The SPR data resulted from binding between the immobilized E-cadherin EC1 domain on COOH chips and the soluble EC1 domain of E-cadherin at different concentrations using **(A)** TBS buffer and **(B)** PBS buffer. The KDs of EC1-to-EC1 binding were 1.45 µM in TBS and 44 µM in PBS.

binding of soluble E-cadherin EC1 domain to the immobilized EC1 domain was evaluated in TBS (**Figure 6A**) and PBS (**Figure 6B**) buffers at different concentrations to determine the KD. The EC1-to-EC1 binding in TBS has a KD of 1.45 μM while in PBS the KD is 44 μM.

The strong binding in TBS compared to in PBS is due to the oligomerization properties of the EC1 domain of E-cadherins in solution. It is proposed that the population of monomeric form of EC1 was higher in TBS than in PBS. Therefore, the binding characteristic the soluble monomer EC1 domain to the immobilized EC1 domain in TBS is higher than that of in PBS. In other words, the oligomers (*e.g.*, dimer, trimer) have lower binding properties to the immobilized EC1 domain because the binding site of the soluble EC1 dimer has been filled due to dimerization. During NMR studies of the E-cadherin EC1 domain, our group found that the EC1 domain of E-cadherin formed aggregates in PBS compared to TBS at a high concentration (*i.e.*, 1.0 mg/ml).

The KD of EC1-to-EC1 binding in PBS (44 μM) has a similar range with previous studies done for homodimerization of EC1-EC2 to EC1-EC2 domains of chicken E-cadherin where the KD is 62 μM at 25°C.[10] It was also found that the KD of homodimerization of EC1-EC1 domain of chicken E-cadherin 110 μM at 37°C. A similar observation was found in homodimerization of EC1-EC2 to EC1-EC2 domains of human E-cadherin in which the KD at 25°C is 156 μM while the KD at 37° is 217 μM. Thus, the results indicate that temperature affects the homodimerization strength of between two EC1-EC2 domains of E-cadherin. It should also be noted that the EC1 domain can interact with EC2 domain; thus, the KD of homodimerization of EC1-EC2 to EC1-

EC2 domains is a combination between binding of EC1-to-EC1 of two molecule (*trans*-binding) and EC1-to-EC2 of two molecules (*cis*-binding) as shown in C-cadherin X-ray structure.

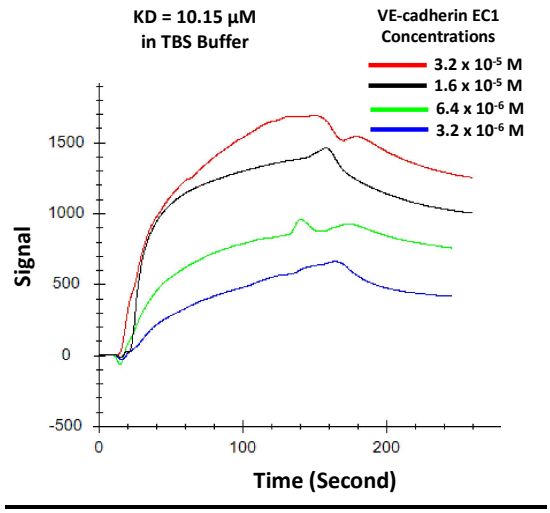

Figure 7. The SPR data resulted from binding between the immobilized EC1 domain of E-cadherin on gold chips and soluble EC1 domain of VE-cadherin in TBS buffer at different concentrations. The KD of EC-to-EC1 binding was 10.15 µM.

3.3. Evaluation of EC1-to-EC1 Binding between E- and VE-cadherin using SPR

The binding between EC1-to-EC1 domains of E- and VE-cadherins was evaluated using gold and carboxylic acid chips. Using the gold chip, the E-cadherin EC1 domain was immobilized by reacting the Cys residue with Au to form a covalent bond (**Figure 2**). Then, various concentrations of the VE-cadherin EC1 domain were flowed through the immobilized E-cadherin EC1 using TBS

running buffer. The concentration-dependent responses from SPR (**Figure 7**) showed that the KD between the EC1-to-EC1 domains of E- and VE-cadherins was 10.15 µM.

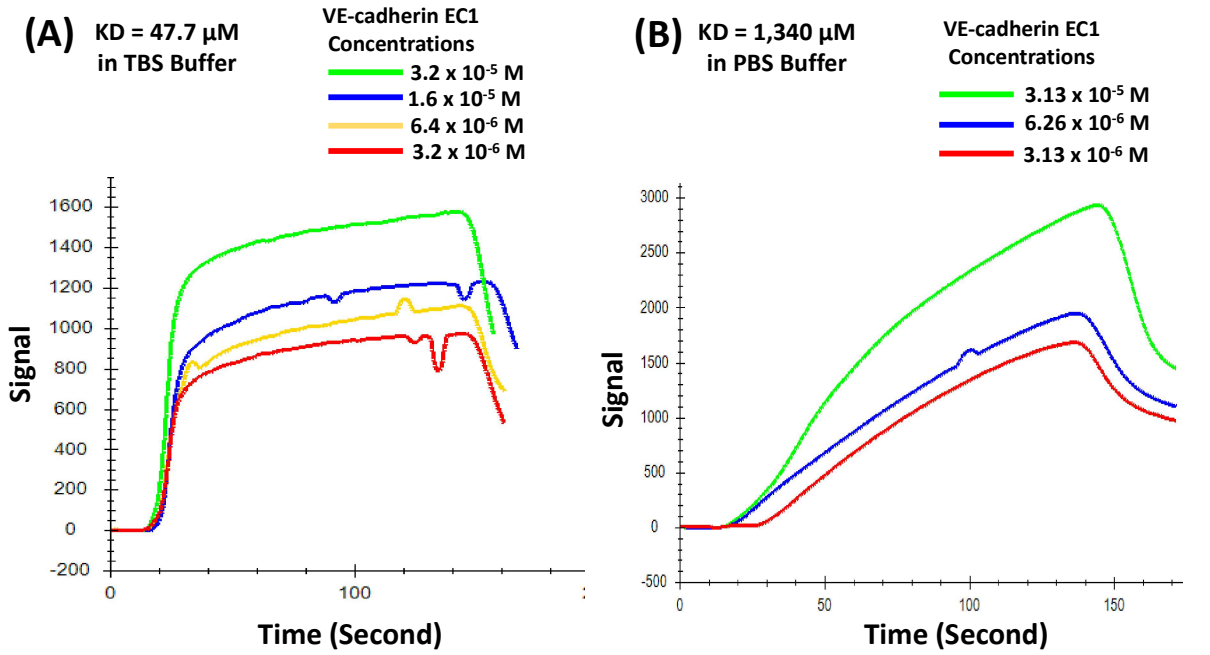

Figure 8. The SPR data resulted from binding between the immobilized EC1 domain of E-cadherin on the COOH chips and the EC1 domain of VE-cadherin in solution at different concentrations using **(A)** TBS buffer and **(B)** PBS buffer. The KDs of EC1-to-EC1 binding were 47.7 µM and 1,340 µM in TBS and PBS buffers, respectively.

To test other methods to evaluate the EC1-to-EC1 binding, carboxylic acid chip was used to immobilize the E-cadherin EC1 domain. The EC1 domain of VE-cadherin at various concentrations in TBS buffer (**Figure 8A**) and PBS buffer (**Figure 8B**) were flowed through the chips. In TBS, the SPR data showed that the KD of EC1-to-EC1 domain binding for E- and VE-cadherins was 47.7 µM (**Figure 8A**). Although the KD value is higher than that in TBS with gold chip (KD = 10.15 µM**; Figure 7**), both KDs in both studies were considered to be in similar magnitude. In contrast, the binding between E- and VE-cadherin EC1 domains was 100 times

weaker in PBS buffer with KD of 1,340 μM. One proposed explanation for low affinity in PBS is due to the oligomerization of the VE-cadherin EC1 domain. The formation of oligomers in solution made the binding side on the VE-cadherin EC1 domain occupied by another EC1 domain. In other words, the population of the monomer of EC1 domain of VE-cadherin is lower in PBS compared to that of in TBS. As shown in mass spectrometry data (**Figure 5**), the VE-cadherin EC1 domain is prone to form stable oligomers (*i.e.*, dimer, trimer, tetramer, pentamer, and heptamer) in PBS.

3.4. Inhibition of EC1-to-EC1 Binding with ADTC5 peptide

ADTC5 peptide has been used to modulate the BBB *in vivo* and enhanced the delivery of small and large molecules including proteins into the brain. The hypothesis is that ADTC5 binds to the EC1 domain of cadherin and inhibit cadherin-cadherin interactions in the BBB. This inhibition increases the porosity of the intercellular junctions for improving diffusion of molecules across the BBB. NMR studies have determined the binding site of ADTC5 at the hydrophobic pocket of "domain swapping" on the EC1 domain of E-cadherin. However, there are no data to show that ADTC5 can inhibit the EC1-to-EC1 homotypic binding between E-cadherin. In addition, the ability of ADTC5 to inhibit mixed interaction between EC1 domains of E- and VE-cadherins can also be evaluated.

Here, the SPR method was used to determine the IC_{50} of ADTC5 to inhibit EC1-to-EC1 binding of E-cadherin as well as a mix between the EC1 domains of E- and VE-cadherins on carboxylic acid chip in PBS buffer (**Figures 9 & 10**). In both studies, the E-cadherin EC1 domain was immobilized on the chip. Then, solutions of different concentrations of ADTC5 in a constant concentration of soluble EC1 domain of E- or VE-cadherin were flowed through the immobilized EC1 domain. The association and dissociation of the eluted EC1 domain were monitored by the

SPR detector. The highest peaks of signal responses from different concentrations of ADTC5 were plotted against the Log of ADTC5 concentrations to determine the IC_{50}.

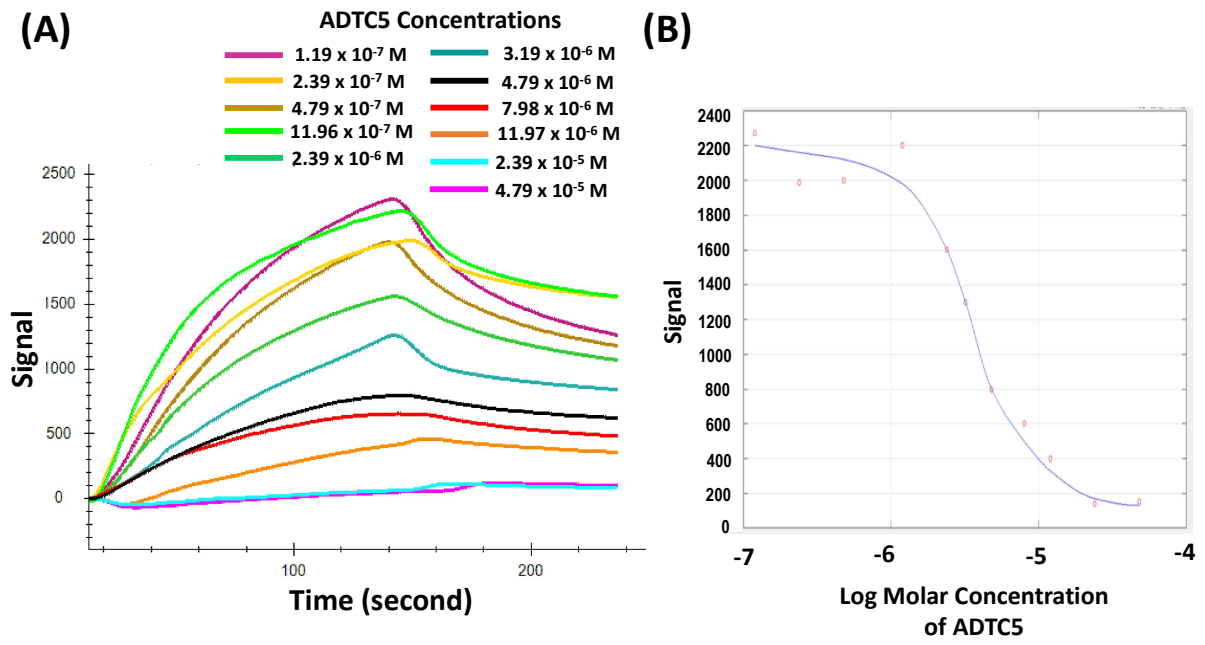

Figure 9. (A) The effects of increasing concentrations of ADTC6 peptide on binding of the soluble E-cadherin EC1 domain to immobilized E-cadherin EC1 domain on COOH chip in PBS buffer. Increasing concentrations of ADTC5 decreased the EC1-to-EC1 binding signals. **(B)** A plot of EC1-to-EC1 binding signals *vs.* log molar concentrations of ADTC5 peptide was used to determine the IC_{50} of ADTC5 to be 4.27 μM.

The binding inhibition of E-cadherin EC1-to-EC1 by ADTC5 showed a decrease in the detected signal as the concentrations of ADTC5 were increased (**Figure 9A**). At very low concentrations of ADTC5 (15 x lower than the concentration of protein), the effect of inhibition by peptide was negligible. At the highest concentrations of ADTC5 (2.3×10^{-5} and 4.79×10^{-5} M), there were no interactions of between the soluble EC1 domain and immobilized EC1. This indicates that there is saturation of the binding sites by ADTC5 to prevent binding. To determine the IC_{50} of ADTC5, a plot between signal response vs. Log ADTC5 concentrations was generated

using MATLAB to give a sigmoidal curve (**Figure 9B**). The IC_{50} value for the inhibition of the E cadherin by ADTC5 is 4.27 μM, where 50% of the EC1 domain binding to immobilized EC1 is inhibited.

A similar study was also carried out to inhibit the EC1-to-EC1 interactions between the E- and VE-cadherins by ADTC5 peptide (**Figure 10**). The signal responses decreased as the

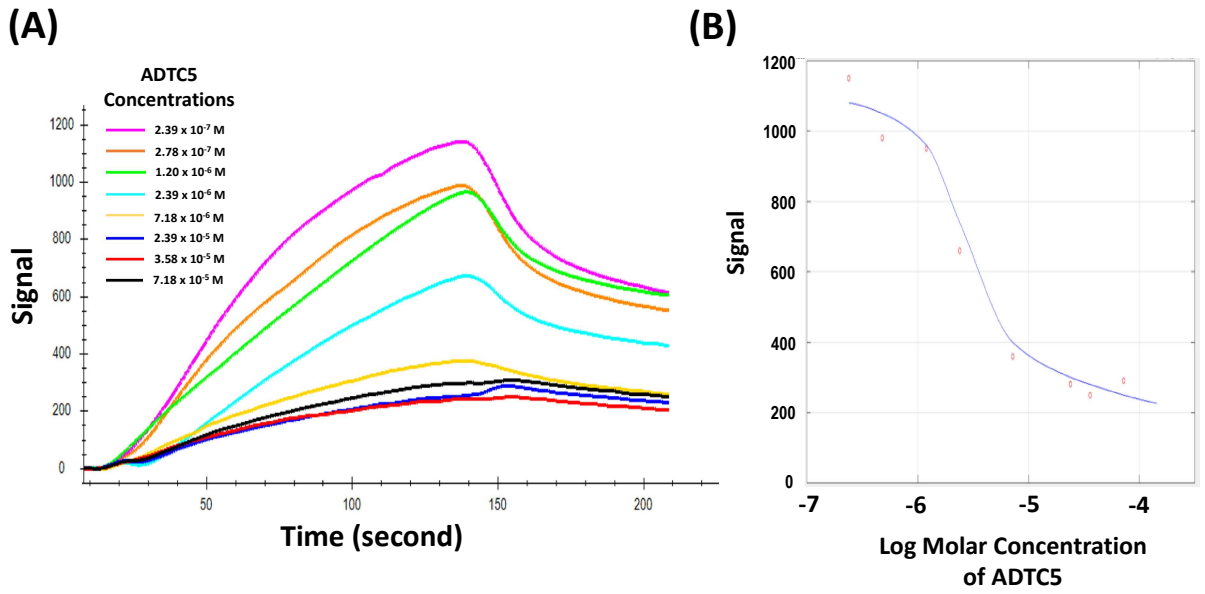

Figure 10. (A) The effects of increasing concentrations of ADTC6 peptide on binding of the EC1 domain of VE-cadherin in solution to immobilized EC1 domain of E-cadherin on COOH chip using PBS buffer. Increasing concentrations of ADTC5 decreased the EC1-EC1 binding signals. **(B)** A plot of EC1-to-EC1 binding signals *vs.* log molar concentrations of ADTC5 peptide was used to determine the IC_{50} of ADTC5 to be 3.27 μM.

concentration of ADTC5 was increased, suggesting that ADTC5 could inhibit mix EC1-to-EC1 binding of E- and VE-cadherin. The plot of signal responses *vs.* Log ADTC5 concentrations gave IC_{50} of 3.27 μM. The results suggest that ADTC5 could also bind to the EC1 domain of VE-cadherin. To test whether ADTC5 binds to the EC1 domain of VE-cadherin, the inhibition of the homophilic EC1-to-EC1 binding of VE-cadherin will be evaluated using SPR in the future.

CHAPTER 4. CONCLUSION

In this study, we have developed SPR method to determine the KDs of binding between homophilic EC1-to-EC1 binding of E-cadherins and heterophilic EC1-to-EC1 binding of E- and VE-cadherins. This study confirmed that ADTC5 binds to the EC1 domain of E-cadherin and can block EC-to-EC1 binding. This study also developed an SPR method to evaluate the IC_{50} of ADTC5 as inhibitor of homophilic or heterophilic binding of EC1-to-EC1 for E- and VE-cadherin. Therefore, this method can be used to evaluate potency and selectivity of new cadherin peptides as inhibitors of E- or VE-cadherin homophilic interactions. While the results showed that ADTC5 inhibited the interaction between E- and VE-cadherin, it could not be determined for certain whether the peptide binds to VE cadherin. In the future, ADTC5 will also be evaluated to inhibit EC1-to-EC1 interaction of VE-cadherin.

One limitation of using the current computer modeling to analyze the SPR data is that it assumes one-to-one fitting of protein-protein interaction. However, it is known that there are oligomers presence in PBS solution of VE cadherin. The same can be true for E cadherin as well and it is not yet known whether the formation of oligomers depends on the concentration of the proteins or the buffer used. When calculating the IC_{50} values, there is a need determine the value at least using three experimental repeats as well as evaluation in different buffers.

In the future, the methods used in this study will be used to determine the interaction properties or KDs of binding between the EC1-to-EC2 domains of E- or VE-cadherins because the mechanism of *cis*-interaction of E- or VE-cadherins involves binding between EC1 and EC2. We have shown previously that HAV peptides bind to the EC1 domain and the NMR studies suggest that they inhibit the EC1-to-EC2 binding for *cis*-interaction of cadherin. Thus, the binding of HAV peptides to the EC1 domain and their IC_{50} to inhibit EC1-to-EC2 domains will be evaluated.

Finally, using computer modeling studies, our group has found new cadherin sequences that are potential selective inhibitors of E- or VE-cadherin; thus, this SPR method will be used to evaluate their activities to inhibit cadherin-cadherin interactions.

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