HOKKAIDO UNIVERSITY

| | |
|---|---|
| Title | Characterization and single chain Fv construction of neutralizing antibody to measles virus |
| Author(s) | JAHAN, MST LUBNA |
| Degree Grantor | 北海道大学 |
| Degree Name | 博士(薬科学) |
| Dissertation Number | 甲第12445号 |
| Issue Date | 2016-09-26 |
| DOI | https://doi.org/10.14943/doctoral.k12445 |
| Doc URL | https://hdl.handle.net/2115/75550 |
| Type | doctoral thesis |
| File Information | JAHAN_MST_LUBNA.pdf |

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

# Doctoral Dissertation

(博士学位論文)

# Characterization and single chain Fv construction of neutralizing antibody to measles virus.

（麻疹ウイルスに対する中和抗体の機能評価と一本鎖 Fv 断片の構築）

**JAHAN MST LUBNA**

**(ジャハン ムスト ルブナ)**

**Laboratory of Biomolecular Science**
**Biomedical and Pharmaceutical Science Course**
**Faculty of Pharmaceutical science**
**Hokkaido University**

**September 2016**

## Abstract

Measles Virus (MV) is a major cause of childhood morbidity and mortality worldwide although a safe and cost-effective vaccine is available. The MV infection is initiated by binding of cellular receptors on the target host cells to hemagglutinin protein of MV (MV-H), a surface glycoprotein responsible for the target cell entry. Several neutralizing antibodies to MV have been developed to different epitopes so far, and most of them target to MV-H protein. Our collaborators (Prof. Yanagi's group) have previously produced and characterized the neutralizing mouse monoclonal antibody (MAb) 2F4, suggesting that the epitope of MAb 2F4 is overlapped with the binding sites of the receptors. The 2F4 MAb is a good candidate antibody with further modification for measles neutralization although the epitopes have not been fully determined yet. The single chain variable antibody fragment (scFv) strategy has become one of the most popular methods in antibody engineering. The aim of this research is to establish scFv of MV neutralizing antibody and reveal its molecular basis for the inhibition of the interaction between MV-H and cellular receptors. For this purpose, the recombinant scFv was prepared from 2F4 MAb, and characterized the biophysical properties using physicochemical techniques. According to the standard protocol in our laboratory, the yield and refolding efficiency were relatively low, about 0.5 mg/L-culture and 1.5%, respectively. Therefore, conditions for some of the purification steps were examined and the yield and refolding efficiency were slightly improved to be about 1.5 mg/L-culture and 4%, respectively. By using the purified 2F4-scFv and MV-H proteins, binding analysis was performed with surface plasmon resonance (SPR), suggesting the recombinant 2F4-scFv interacts with MV-H in concentration dependent manner with lower affinity ($K_D$ of 5.0 μM) than Fab as well as than cellular receptors.

Next, we tested whether binding of scFv is affected by the SLAM or nectin-4. The results indicated the binding of scFv was inhibited by nectin-4 and also by SLAM. To determine whether 2F4-scFv can inhibit membrane fusion mediated by MV-H binding to cellular receptor, I performed conventional fusion assay using receptor expressing cells, suggesting 2F4-scFv has ability to inhibit the MV-H and F- mediated syncytia formation as like intact 2F4 MAb. My research results reveal that scFv specifically binds to the MV-H at μM level of $K_D$ value and is able to interfere the binding of MV-H to its cellular receptor, furthermore it can inhibit membrane fusion by blocking receptor binding sites on MV-H, and account for the hypothesis that the 2F4 uses the receptor binding sites as a neutralizing epitope. I expect that the newly prepared 2F4-scFv will enable us to further investigate the inhibitory mechanism of cell entry via MV-H by 2F4-scFv, thereby to design an effective vaccine for MV infection.

# Contents

**Abbreviations** 5

**Introduction** 7
- Measles virus (MV) 7
- Cellular receptors for the measles virus 9
- Membrane fusion mediated by MV-H and F 10
- Epitopes for anti-measles monoclonal antibodies 11
- Neutralizing monoclonal antibody (MAb), 2F4 11
- The single chain fragment variable (scFv) of the antibody 16
- Surface Plasmon Resonance (SPR) 17
- Aim of the study 20

**Materials and Methods** 21
- Materials 21
- Methods 25

**Results** 32
- Construction of the expression vector of the 2F4-scFv 32
- Expression and purification of recombinant 2F4-scFv Proteins 32
- Binding kinetics of 2F4-scFv, 2F4 Fab, SLAM and nectin-4 to the MV-H 42
- Effect of receptors on the 2F4-scFv binding to MV-H 47
- Effect of the 2F4-scFv on the membrane fusion 49

**Discussion** 51

**Conclusion** 55

**References** 56

**Acknowledgement** 66

# Abbreviations

| | |
|---|---|
| BSA | Bovine serum albumin |
| CBB | Coomassie brilliant blue |
| CDC | Centers for Disease Control and Prevention |
| CDR | Complementarity determining region |
| DMEM | Dulbecco's Modified Eagle Medium |
| Ed | Edmonston strain |
| EDTA | Ethylenediamine-*N,N,N',N'*-tetraacetic acid |
| EGFP | Enhanced green fluorescent protein |
| F | Fusion |
| Fab | Antigen-binding fragment |
| FBS | Fetal bovine serum |
| GFP | Green fluorescent protein |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| H | Hemagglutinin |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| $k_a$ | Association rate constant |
| $k_d$ | Dissociation rate constant |
| $K_D$ | Dissociation constant |
| LB | Luria-Bertani |
| MAb | Monoclonal antibody |
| MV | Measles virus |

| | |
|---|---|
| NE | Neutralizing epitope |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| PDB | Protein Data Bank |
| PEI max | Polyethyleneimine max |
| PVRL4 | Poliovirus receptor-related 4 |
| RBS | Receptor-binding site |
| rpm | Rotation per minute |
| scFv | single chain fragment variable |
| SDS-PAGE | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| SEC | Size exclusion chromatography |
| SLAM | Signaling lymphocyte activation molecule |
| SPR | Surface plasmon resonance |
| SSPE | Subacute sclerosing panencephalitis |
| Tris | Tris (hydroxymethyl) aminomethane |
| VH | Variable regions of heavy chain |
| VL | Variable regions of light chain |
| WHO | World Health Organization |
| YT | Yeast-tryptone |

# Introduction

**Measles virus (MV)**

Measles virus (MV) is a major cause of childhood morbidity and mortality all over the world (1,2). World Health Organization (WHO) reported that approximately 114,900 people died from measles globally in 2014 although an effective vaccine is available (http://www.who.int/). Centers for Disease Control and Prevention (CDC) reported that a large number of measles cases, such as 667 cases from 27 states, were found in the United States in 2014, and a large measles outbreak, including 189 infected people from 24 different states occurred in 2015 (http://www.cdc.gov/). Thus, MV infections remain possible threat to global health. The clinical symptoms for measles virus infection include fever, cough, photophobia and a symptomatic red rash over most part of the body (3). Long-term persistence of measles virus leads to encephalitis and subacute sclerosing panencephalitis resulting from infection of the central nervous system (4). In several developing countries, measles has become a serious health threat for human due to low vaccination coverage.

Measles virus belongs to the genus, *Morbillivirus* in the family *Paramyxoviridae* (3,5). The virus possesses a nonsegmented negative-sense RNA genome encoding genes for the proteins named the nucleocapsid protein (N), a phosphoprotein (P), virulence factors (C and V), a matrix protein (M), a membrane fusion protein (F), a receptor binding hemagglutinin (H) and an RNA polymerase (L) (Fig. 1). Of them, the hemagglutinin (H) and fusion (F) proteins are expressed on the viral envelope, highly glycosylated in nature (Fig. 2) and are involved in the MV entry to the host cells. Thus, both proteins became neutralizing targets.

**Figure 1: Schematic representation of the genome structure of measles virus.**
MV has a nonsegmented negative-strand RNA genome, which encodes N, P, M, F, H and L proteins. The P gene encodes V and C genes, respectively, which are alternatively spliced.

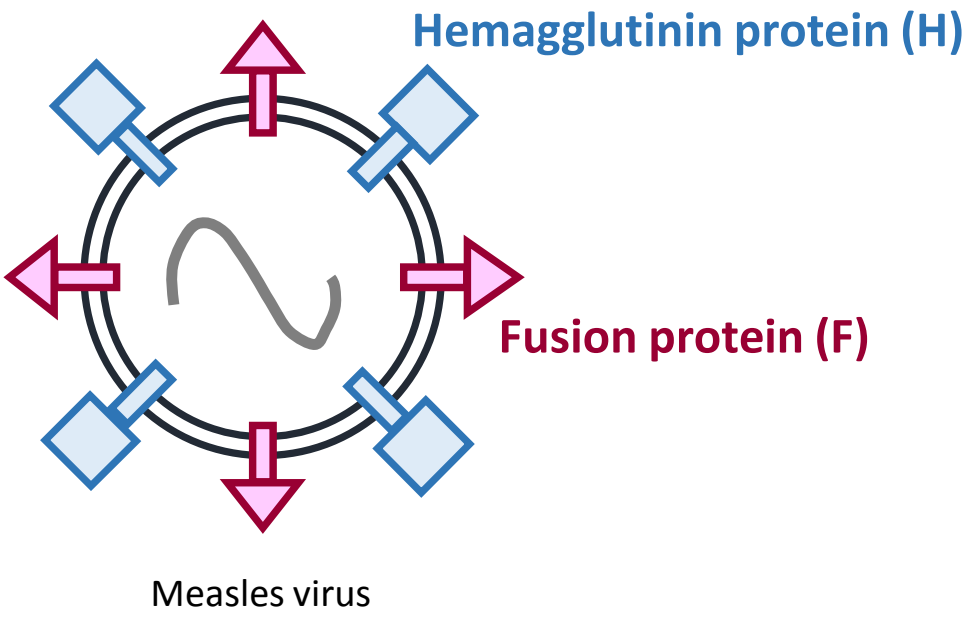


**Figure 2: Cartoon representation of the measles virus.**
Envelope glycoproteins, hemagglutinin (H) and fusion (F) proteins are highlighted.

**Cellular receptors for the measles virus**

Until now, there are three cellular receptors for measles have been identified, including the signaling lymphocyte activation molecule (SLAM), the epithelial protein nectin-4 and the complement regulatory protein CD46 (6-10). Both wild-type and vaccine strains of measles viruses can use SLAM and nectin-4 for the membrane fusion with host cells, whereas only vaccine strain of the Edmonston virus can use CD46 (7,11,12).

SLAM is expressed on immune cells, like thymocytes, activated lymphocytes, mature dendritic cells, macrophages and platelets (13), and can be a marker for the most primitive hematopoietic stem cells (14). It can interact with another SLAM molecule present on an adjacent cell as a self-ligand with a low affinity of approximately 200 μM (15). The immunosuppressive nature of measles virus is due to the use of SLAM as a cellular receptor (11). SLAM is a member of the immunoglobulin (Ig) superfamily, a type I membrane protein that possesses an N-terminal signal sequence, two Ig-like domains (V-set and C2-set according to standard Ig-like domain category), a transmembrane region, and a cytoplasmic tail (16). Each Ig-like domain consists of about 110 amino acid residues and only the membrane-distal V-set domain is important for MV-H binding (17).

The nectin-4 is expressed on epithelial cells, and various tissues including the human airway, skin, lung, prostate and stomach (18,19). The protein is a member of the $Ca^{2+}$-independent immunoglobulin-like cell-cell adhesion molecules, which contains nine members (nectin-1 to nectin-4 and Necl-1 to Necl-5) (20). They contain three immunoglobulin-like domains (V-C-C sets), playing an important role in mediating cell-cell adhesion (21,22).

The complement-regulatory protein, CD46, is expressed in all kinds of human cells and first identified as a cellular receptor for the Edmonston strain of MV (9,10). The extracellular region of CD46 consists of four short consensus repeats (SCR1–SCR4), and only the membrane-distal, N-terminal SCR1 and SCR2 play a role in the interaction with MV-H (23).

**Membrane fusion mediated by MV-H and F**

It is thought that the binding of the H protein to a receptor leads to the membrane fusion between the viral envelope and the host cell plasma membrane mediated by F protein. We have determined the MV-H and SLAM complex structure of different dimer of dimer conformations (Fig. 3) (17). The structure shows that the MV-H homodimer structure is highly covered by the potential N-linked sugars and limited region is exposed, including the binding site for SLAM as well as that for nectin-4 (Fig. 4). Furthermore, the epitopes for most monoclonal antibodies to MV-H (24-27) have been shown on the MV-H homodimer structure (Fig. 4). They represent that all receptors and most of the neutralizing antibodies binding sites locate limited regions on the MV-H, indicating a single antibody can be effective to inhibit measles virus infection mediated by different receptors. Based on the structure of dimer of dimers of MV-H (Fig. 3), we previously proposed the model for the sequential events during measles virus-induced membrane fusion (17,28). The MV-H tetrameric structure is formed together with the MV-F trimer on the viral envelope. When the SLAM binds with MV-H, conformational change of the SLAM occurred. The interaction between SLAM and MV-H reduces the distance between the viral and host membranes (29-32), providing a scaffold for the structural change of MV-F and the configuration of the

dimer of dimers may shift, leading to change the orientation of the stalk regions, allowing MV-F to refold and to effectively interact with the target cell membrane, ultimately lead to membrane fusion (17). Although neutralizing antibodies against each of the viral envelope glycoproteins are produced, H protein-specific antibodies are predominant for the inhibition of MV infection (27,33,34). Therefore, H protein comes into a spotlight as a neutralizing target.

**Epitopes for anti-measles monoclonal antibodies**

Almost all of the measles vaccines were isolated about a half a century ago, and were composed of live attenuated MV strains. There are 24 genotypes for MV, and all vaccine strains are classified into the single genotype (genotype A) (1). Although there are differences in the genotypes present in different countries or regions, measles vaccines have been effective till now. Consequently, the effectiveness of measles vaccine suggests that MV does not undergo a major antigenic drift. However, several studies indicate that antigenic variations occur among currently circulating MV strains, and the efficacy of vaccination could be possibly affected by this antigenic variations (35-40). To date, several neutralizing epitopes (NEs) on MV-H were identified, which likely locate at the receptor-binding site (RBS) or a site involved in the interaction with the F protein (41). Several neutralizing antibodies with the amino acid residues consist of epitope regions have been well documented by Bouche *et al*. (27). The epitopes and antibodies were summarized in Table 1 and mapped on the H protein structure (Fig. 4).

**Neutralizing monoclonal antibody (MAb), 2F4**

Our collaborators generated and characterized a mouse monoclonal antibody

(MAb) 2F4 (42). The MAb 2F4 was produced using a cell line expressing the H protein of the wild-type IC-B strain as an antigen. They performed the infection assay using eight different recombinant MVs and the result revealed that the MAb 2F4 could efficiently neutralize the infection of all eight rMVs to both SLAM- and nectin-4-expressing cells. The results indicated that the 2F4 may have ability to block the viral entry mediated via MV-H binding to both SLAM and nectin-4. The epitope recognized by 2F4 is tentatively termed epitope *vii* although the epitope has not been completely determined yet. Based on the results, they hypothesized that the epitope *vii* is overlapped with the binding site of the cellular receptors. Therefore, the receptor binding sites recognized by both SLAM and nectin-4 are mapped as epitope *vii* on the MV-H protein structure as well as the identified antigen binding sites as summarized in Table 1 (Fig. 4).

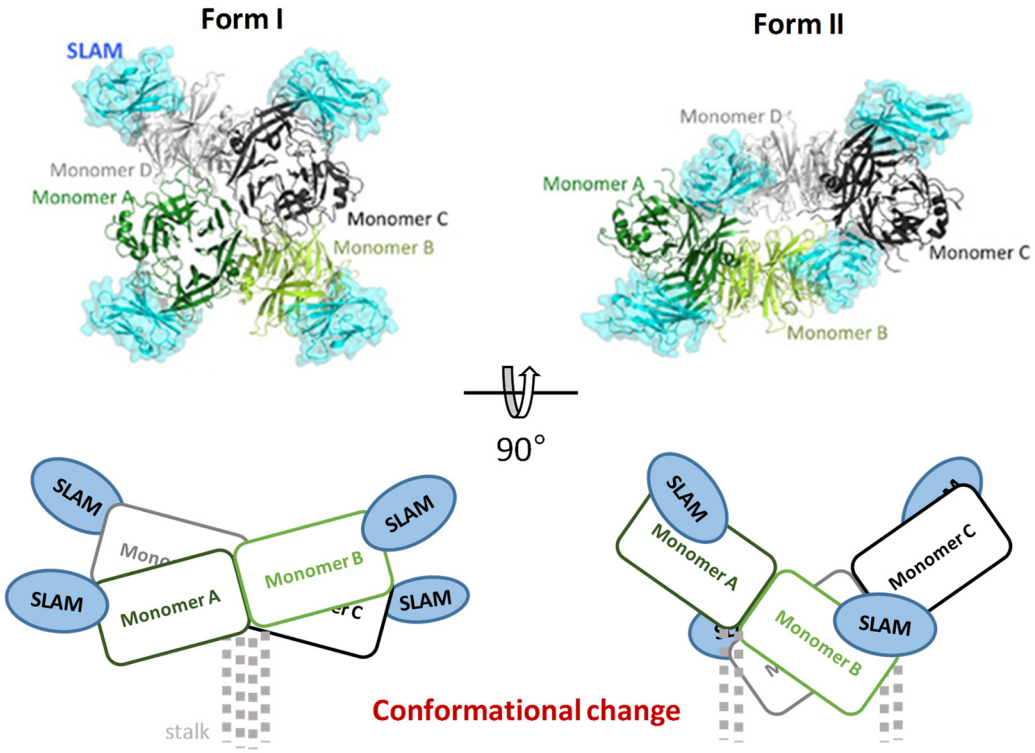


**Figure 3: Different dimer of dimer (tetramer) forms of MV-H bound to SLAM.**
(Top) Form I and II of MV-H dimer of dimers (tetramer) shown in cartoon models with bound to SLAM shown in surface and cartoon models (cyan). MV-H monomers A (dark green) and B (light green) form one dimer, whereas monomers C (black) and D (gray) form another dimer. (Bottom) Schematic representation of Form I and II. The view is rotated by 90° around a horizontal axis. Figures were modified from references (17, 28).

**Table 1: List of the epitopes and monoclonal antibodies.**

| Epitope name | Antigenic region or sites on MV-H | Monoclonal antibody |
|---|---|---|
| *Iv* | 233–240 | E185, BH1 |
| *V* | 302 | E39 |
| *Vi* | 309–318 | E103, I-29, BH38 |
| *I* | 380–400 | E81, B5, BH6, BH21, BH216 |
| *II* | 473–477 | E128 |
| *vii* | 464, 482, 483, 500, 505, 524, 543, 548, 550 (receptor binding sites) | 2F4 |

Since the antigenic sites for epitope *vii* was not fully determined yet, the binding residues identified from the complex structures between MV-H and SLAM or nectin-4 were listed.

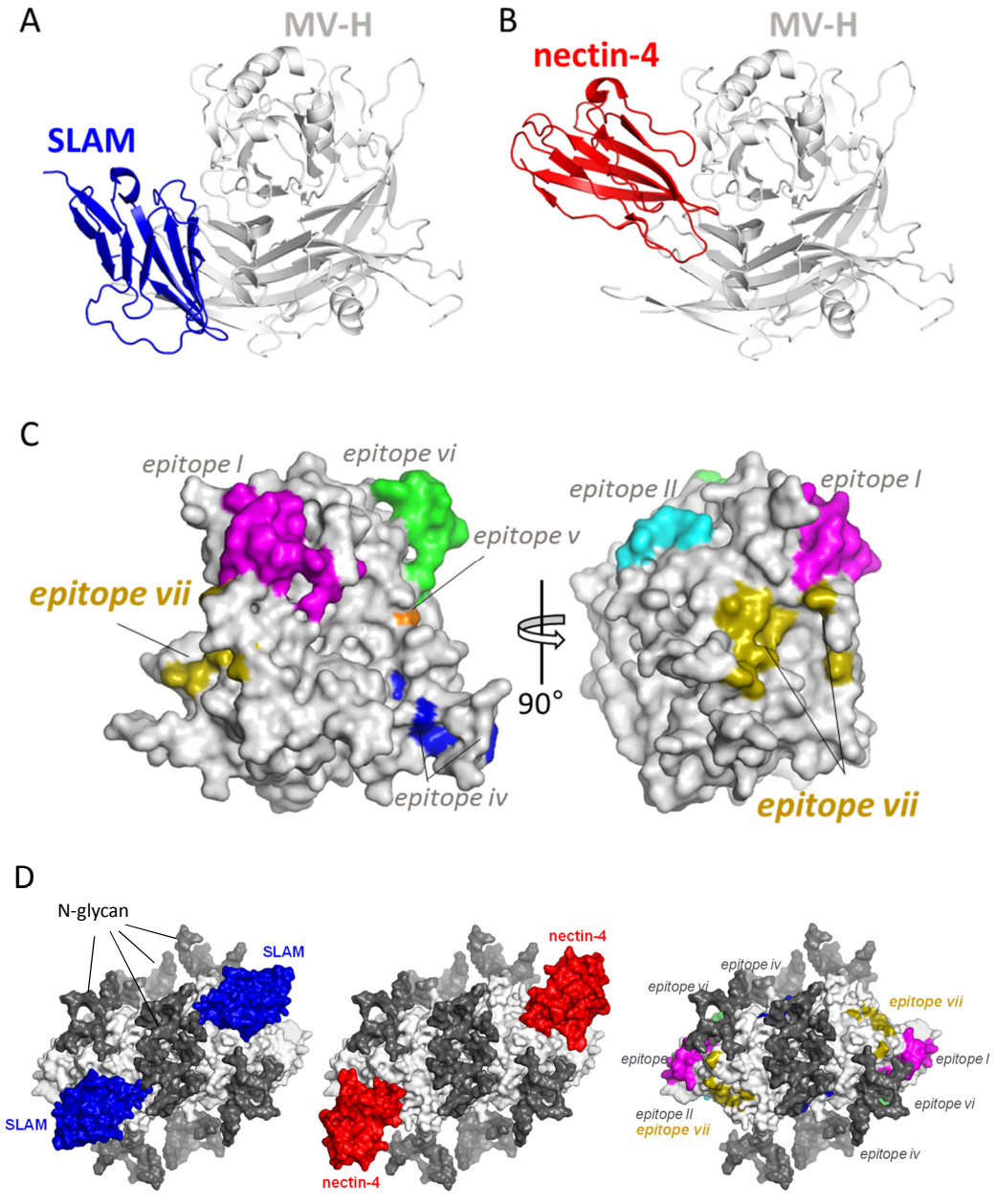


**Figure 4: The complex structures of MV-H with receptors and the location of the epitopes on the MV-H protein.** (A) Complex structure of MV-H and SLAM (PDB ID: 3ALZ). (B) Complex structure of MV-H and nectin-4 (PDB ID: 4GJT). (C) The location of the epitopes for neutralizing antibodies summarized in Table 1. Surface representation of the MV-H. The left panel shows a view of the same direction of A and B. The right panel shows a view rotated by 90° around a vertical axis. (D) Surface representation of the MV-H homodimer with SLAM (left), with nectin-4 (middle) and the location of the epitopes for neutralizing antibodies (right). MV-H is shown in white, potential N-linked glycan is shown in black (gray and light gray depending on the depth).

**The single chain fragment variable (scFv) of the antibody**

Monoclonal antibodies (MAbs) become one of the most popular therapeutic tools including virus neutralization (43,44). However, intact antibodies are large in size, sometimes show high level of immunogenicity, and the production is poorly economical. Hence, small antibody fragments are recently being used as alternatives to monoclonal antibodies in therapeutic applications (45,46). In the field of antibody engineering, the single chain fragment variable (scFv) antibody strategy has become one of the most popular method because of its advantages in comparison to full length antibodies such as lower level of immunogenicity due to lacking of Fc region, and small molecular size, facilitating more efficient penetration into the tissue (45-47). Although scFv is the smallest unit of immunoglobulin molecule, it has antigen-binding activities. An antibody in scFv format consists of variable regions of heavy ($V_H$) and light ($V_L$) chains, connected with a flexible peptide linker (Fig. 5). It is possible to improve the properties of scFv by using protein engineering approach, such as increase of affinity and alteration of specificity (48). To facilitate correct folding, the length of the flexible peptide linker is the critical point (49). Not only the length but also the amino acid composition of the linker play an important role in successful design of scFv. To avoid insertion of peptide between the variable domains during the protein folding, hydrophilic residues must be included into peptide linker (50). Nowadays, $(GGGGS)_3$ linker, consist of 15 amino acid residues including four glycines and one serine repeated three times, is most widely used, which was designed on the basis of the structures of Fv domain determined by X-ray crystallography as well as computer-aided analysis (49,51). The linker has two function; one is connection between the N- and C-termini of individual variable regions, the other is to tighten the $V_H$ and $V_L$ domains without

interfering the interaction between Fv domains. Due to lowest molecular weight and shortest side chain of amino acid, glycine is able to increase the flexibility of the side chain, while serine, sort of hydrophilic amino acid, allows to increase the solubility of the linker (52). Based on these facts, $(GGGGS)_3$ is taken into consideration to connect Fv domains (Fig. 5).

**Surface Plasmon Resonance (SPR)**

SPR is a powerful tool to analyze interaction between molecules and the systems can monitor molecular interactions in real time (53). To study the interaction between two binding partners, the ligand is immobilized to the surface of thin gold layer, called sensor chip, and the analyte in mobile state is passed over the sensor chip surface in a continuous flow. The detection principle is based on the surface plasmon resonance that monitors the angle changes of the reflected light, corresponding to the changes in refractive index. The intensity of the reflected light is converted to 'resonance signal' or 'response'. A plot of response against time, called sensorgram is obtained as a data (Fig. 6).

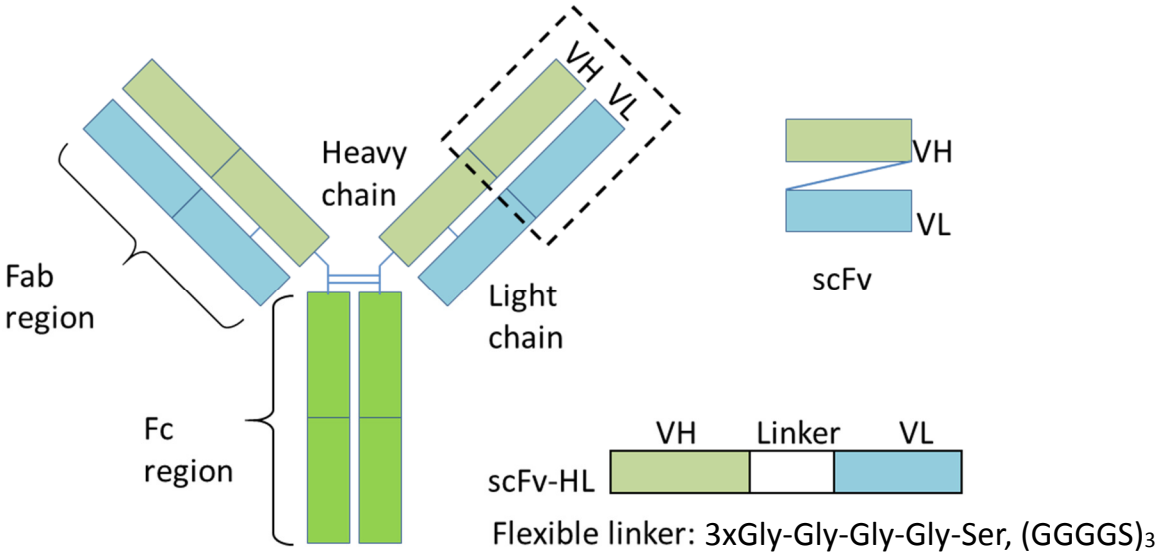


**Figure 5: Schematic representation of the full length and the scFv antibodies.**
In the scFv, $V_H$ and $V_L$ domains are linked with the glycine-serine flexible linker $(GGGGS)_3$.

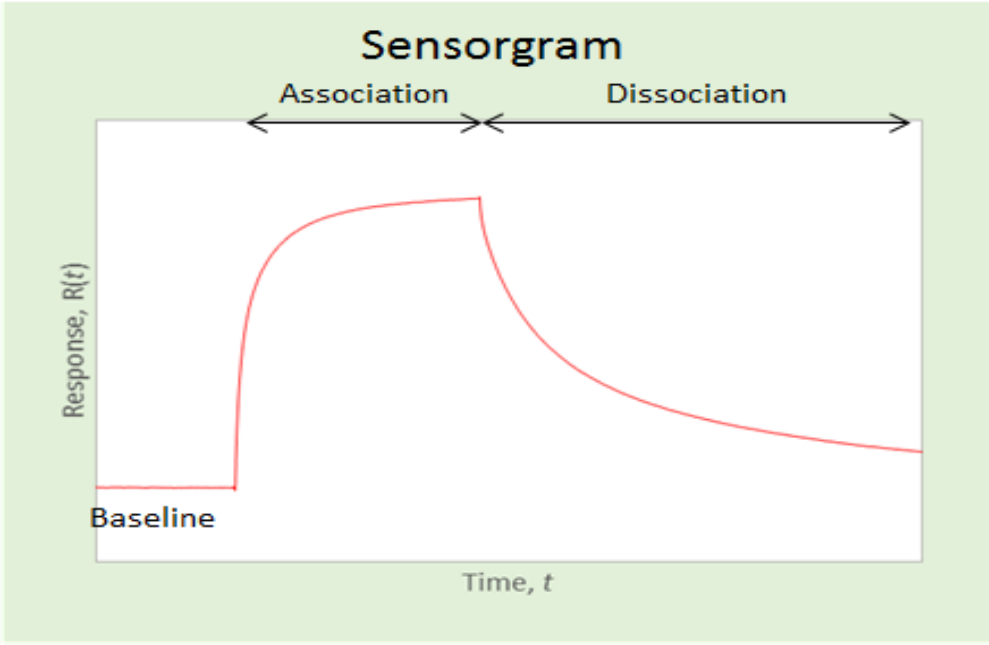


**Figure 6: Surface plasmon resonance (SPR)**
The representative sensorgram obtained from kinetic analysis.

**Aim of the study**

The aim of this study is to reveal the molecular basis for the inhibition of the interaction between MV-H and cellular receptors, thereby to develop effective vaccine and anti-viral drugs to measles. For this purpose, I focused on the neutralizing antibody MAb 2F4, I constructed and purified recombinant scFv of MAb 2F4. Using the recombinant proteins, I analyzed the binding kinetics of antibody and cellular receptors to the MV-H by SPR technique. Furthermore, competitive binding analysis and conventional fusion assay using receptor expressing cells were performed whether the scFv can inhibit the binding of MV-H to the cellular receptors and the membrane fusion mediated by the interaction between MV-H and cellular receptors. I expect that the results in this study will provide understanding the inhibitory mechanism of MV entry into the host cells, and new avenue to eradicate measles by developing effective vaccine and anti-viral drug against MV infection.

# Materials and Methods:

## Materials:

**Table 1: PCR primers for the construction of scFv expression vectors.**

| Name | Sequence | Restriction site |
|---|---|---|
| *For HL* | | |
| VH-Fw | 5′-GCGAAGACGTACATATGGAGGTGCAGCTGGTGGAG-3′ | NdeI |
| VH-linker-Rv | 5′-*GGATCCACCACCTCCACTGCCGCCACCACCAGACCCACCGCCGCCT*GGTGTCGTTTTGGCTGAGG-3′ | |
| VL-linker-Fw | 5′-*GGCGGCAGTGGAGGTGGTGGATCC*ACACAGACTCCAGCCTCCC-3′ | |
| VL-Rv | 5′-GCGGAATTCTTACCGTTTGATTTCCAG-3′ | EcoRI |
| *For LH* | | |
| VL-Fw | 5′-CATTCATATGACACAGACTCCAGCCT-3′ | NdeI |
| VL-linker-Rv | 5′-*GGATCCACCACCTCCACTGCCGCCACCACCAGACCCACCGCCGCCC*CCGTTTGATTTCCAGCTTGG-3′ | |
| VH-linker-fw | 5′-*GGCGGCAGTGGAGGTGGTGGATCC*GAGGTGCAGCTGGTGGAGTCTGGG -3′ | |
| VH-Rv | 5′-GCGGAATTCTTATGGTGTCGTTTTGGC-3′ | EcoRI |

Underlined sequence indicates restriction site. Italic sequence indicates flexible linker region encoding GGGGS repeat. PCR primers were synthesized by Eurofins Genomics.

### Plasmids

pET22b (Novagen), 2F4-$V_H$/pGEM-T-vector and 2F4-$V_L$/pGEM-T-vector for scFv construction

MV-H/PS-pCA7, MV-F/pCXN2 and EGFP/pCA7 for fusion assay

### Cells

*E. coli*, DH5α (TOYOBO) competent cells

*E. coli* expression strain BL21(DE3)CodonPlus-RIL competent cells (Agilent)

Vero/SLAM, Vero/nectin-4, cultured in DMEM supplemented with 10 % FBS, at 37°C with 5% $CO_2$

**Media**

**LB-Agar plate:**

20 g/L LB Broth (Sigma-Aldrich) and 20 g/L Agar, autoclaved.

**2xYTmedium:**

16 g/L Tryptone, 10 g/L Yeast Extract and 6 g/L NaCl, autoclaved.

**For Cell culture**

Dulbecco's Modified Eagles Medium (DMEM) (Wako)

**Protein**

MV-H, SLAM, nectin-4, Fab and MAb 2F4, prepared in our laboratory

**Buffers**

Resuspension buffer: 50 mM Tris-HCl pH 8.0, 100 mM NaCl

6 M guanidine HCl containing buffer (6 M GuHCl buffer): 6 M GuHCl, 50 mM Tris-HCl pH 8.0, 10 mM EDTA.

Triton wash buffer: 50 mM Tris-HCl pH 8.0, 100 mM NaCl, 0.5% Triton X-100

Refolding buffer: 400 mM L-Arginine, 100 mM Tris-HCl pH 8.0, 2 mM EDTA, 3.73 mM Cystamine, 6.34 mM Cysteamine

SEC running buffer: 20 mM Tris-HCl pH 8.0, 100 mM NaCl

HBS-P buffer: 0.01 M HEPES pH 7.4, 0.15 M NaCl, 0.005% v/v Surfactant P20

**Other materials and equipments**

Agarose gel/DNA purification kit (QIA quick Gel Extraction Kit) (QIAGEN)

Restriction enzyme EcoRI (TaKaRa) and NdeI (NEB)

AmpliTaq Gold master mix (ABI)

KOD-Plus- DNA polymerase Ver. 2 (TOYOBO)

Ligation High Ver. 2 (TOYOBO)

BigDye terminator ver 3.1 Cycle Sequencing Kit (Applied Biosystems).

The sequence analysis samples were prepared in Hi-Di formamide (Applied Biosystems).

AKTA purifier UPC 10 (GE Healthcare)

HiLoad 26/60 Superdex75, Prep grade (GE Healthcare)

Biacore3000 (GE Healthcare)

CM5 Sensor Chip (GE Healthcare)

Polyethyleneimine (PEI) max (Polysciences)

$CO_2$ incubator (Panasonic)

Inverted System Microscope IX71 with IX2-RFA Fluorescence Illuminator (OLYMPUS)

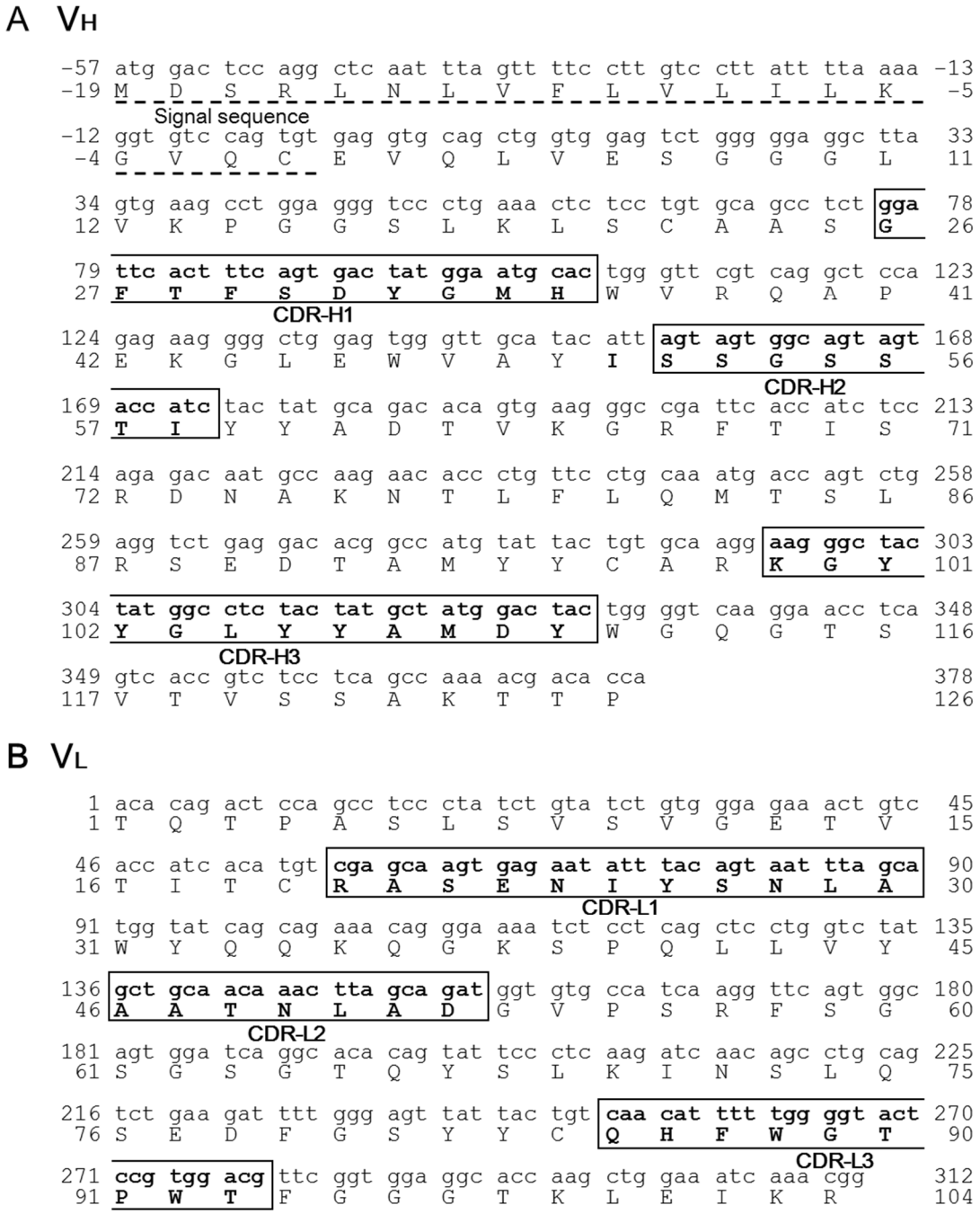


**Figure 7: Sequence of $V_H$ and $V_L$ domains of MAb 2F4.**
The DNA and amino acid sequences of the variable domain in heavy (A) and light (B) chains. Broken line below variable domain of heavy chain indicates signal sequence. Every CDR sequence is boxed off.

# Methods

### Construction of 2F4-scFv/pET22b as an expression vector

First, I started to construct the 2F4-scFvs in pET22b for the recombinant protein expression since the genes encoding $V_H$ and $V_L$ domains of MAb 2F4 were already cloned into the pGEM-T vector and the sequences were also determined in our laboratory (Fig. 7). To construct the scFvs, I utilized splicing overlap extension PCR method (54) via the sequence encoding glycine-serine $(GGGGS)_3$ flexible linker. The PCR was carried out using KOD-Plus DNA polymerase (TOYOBO) according to the manufacturer's instruction. The template cDNAs for variable domains of heavy and light chains in the pGEM-T vectors were amplified by PCR with following primer pairs summarized in Table 1: VH-Fw and VH-linker-Rv, VL-linker-Fw and VL-Rv for scFv-HL, VL-Fw and VL-linker-Rv, VH-linker-Fw and VH-Rv for scFv-LH, respectively. The primers, named -linker-, contain the partial sequence for the $(GGGGS)_3$ linker to assemble both variable domains. The amplified fragments encoding 2F4-$V_H$ and 2F4-$V_L$ were used as templates for the second round PCR reaction. The primer sets of VH-Fw and VL-Rv, or VL-Fw and VH-Rv for HL or LH construct were used. The assembled fragments were then digested by the restriction enzymes, NdeI and EcoRI, purified from the agarose gel using DNA purification kit (QIAquick Gel Extraction Kit) and ligated into the NdeI-EcoRI site of the pET22b vector using Ligation High Ver. 2. The ligation products were transformed into *E. coli* DH5α competent cells, the insertion of the desired gene was confirmed by the digestion of restriction enzymes, followed by DNA sequencing with ABI3100 sequencer (Applied Biosystems). The resultant plasmids were designated as 2F4-scFv-HL/pET22b and 2F4-scFv-LH/ pET22b for the expression of $V_H$-$V_L$ and $V_L$-$V_H$ orientations,

respectively.

**Expression of recombinant 2F4-scFv and preparation of the inclusion body**

The recombinant 2F4-scFvs were expressed by transforming *E. coli* BL21(DE3)CodonPlus-RIL with 2F4-scFv-HL/pET22b or 2F4-scFv-LH/pET22b. For transformation, 1μL of the plasmid was added to the BL21(DE3)CodonPlus-RIL competent cells, the cells were incubated on ice for 20 min, then subjected to heat shock for 40 sec at 42 °C, after heat shock the cells were incubated for 2 min on ice, 300 μL of 2×YT medium was then added to the cells and the cells were incubated at 37 °C with shaking for 60 min. 200 μL of the incubated solution was then spread onto LB agar plate containing 100 μg/mL of ampicillin and the plate was incubated for overnight at 37 °C. For pre-culture, one colony was picked up and added to the 5 mL of 2×YT medium with 100 μg/mL of ampicillin followed by shaking for overnight at 37 °C. The pre-cultured medium (10 mL for two tubes) was then added to 1 L of 2×YT medium with 100 μg/mL of ampicillin and the cells were cultured with shaking at 150 rpm at 37 °C. The expression of 2F4-scFv was induced by addition of 1 mM IPTG at OD600 = 0.8-1.0 and incubated at 37 °C for further 6 hours. After induction, the cells were harvested by centrifugation at 5,000 × g for 10 min. Cell pellet was re-suspended in resuspension buffer, disrupted by sonication and separated by centrifugation at 8,000 rpm for 10 min. The scFv was obtained in an insoluble fraction, and the pellet was washed repeatedly (four times) with Triton wash buffer, and subsequently wash four times with resuspension buffer to remove Triton. The resultant inclusion body was solubilized in 6 M guanidine HCl containing buffer (6 M GuHCl buffer). The expression of 2F4-scFv and the purity of the inclusion body was checked by 15%

SDS-PAGE.

**Refolding and purification of 2F4-scFv from inclusion body**

Solubilized inclusion body was refolded by dilution method. Firstly, DTT (10 mM) was added to the solubilized inclusion body and incubated for 1 hour at room temperature. The inclusion body was slowly diluted with the ice-cold refolding buffer (400 mM L-Arginine, 100 mM Tris-HCl pH 8.0, 2 mM EDTA, 3.73 mM Cystamine, 6.34 mM Cysteamine) to roughly four-fold dilution. The diluted inclusion body was added to the ice-cold refolding buffer to become around 1.5 μM at final concentration. The resultant diluted solution was incubated at 4 °C for 3 days by gently stirring to allow denatured protein to be properly folded. The refolded protein solution was concentrated using VIVAFLOW50 system (Sartorius) to the appropriate volume, usually till the aggregates appeared. Subsequently the buffer was exchanged to the SEC running buffer to remove excess amount of L-Arginine and separate soluble fraction by centrifugation at 30,000 × g for 30 min. The soluble fraction was applied onto the size exclusion chromatography (SEC) with the HiLoad26/60 Superdex75 Column which was equilibrated with SEC running buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl). Unless the different conditions were mentioned in the result section, I followed this protocol described above. The refolding efficiency was calculated by using the following equation (*eq 1*).

$$\text{Refolding efficiency (\%)} = \frac{\text{Refolded protein (g)}}{\text{Inclusion body applied to refolding (g)}} \times 100 \qquad (eq\ 1)$$

**Kinetic analysis by SPR**

To determine the kinetic parameters between two interacts by analyzing the data using 1:1 Langmuir binding model. The simplest situation of the interaction between two molecules (A and B) are described as the equation 2 (eq 2). $k_a$ is the association rate constant, while $k_d$ is the dissociation rate constant. During association and dissociation, the data will be analyzed using the equations for association state (*eq 3*) and dissociation state (*eq 4*). C is the analyte concentration, and $R_{max}$ is the maximal capacity of the sensor chip surface. From the fitting, therefore, the association and dissociation rate constants $k_a$ and $k_d$ will be determined. When the reaction reaches at equilibrium state, the dissociation constant $K_D$ (and association constant $K_A$) can be calculated by the equation 5 (*eq 5*).

1:1 binding model

$$A + B \underset{k_d}{\overset{k_a}{\leftrightarrows}} AB \qquad (eq2)$$

Association state

$$R(t) = \frac{k_a.C.R_{max}}{k_a.C+k_d}[1 - exp\{-(k_a.C + k_d)\}t] \qquad (eq3)$$

Dissociation state

$$R(t) = R_0.exp(-k_d.t) \qquad (eq4)$$

At equilibrium state,

$$K_D = \frac{1}{K_A} = \frac{k_d}{k_a} \qquad (eq5)$$

**Kinetic analysis of the interaction of antibodies or receptors to MV-H.**

By using the purified 2F4-scFv, 2F4 Fab, SLAM and nectin-4 as an analyte and MV-H as a ligand, kinetic binding analysis was performed with SPR. Recombinant proteins of MV-H head domain, ectodomain of SLAM and nectin-4 were prepared in our laboratory. Fab fragment of 2F4 MAb was prepared by digesting with papain. The MV-H head domain was biotinylated according to the manufacturer's instruction. The biotinylated MV-H was immobilized on the sensor chip CM5 at roughly 1000 RU by amine-coupling. The analyte was dissolved in the HBS-P buffer and 30 μL of the analyte at various concentrations was injected at a flow rate of 25 μL/min over the immobilized MV-H protein or the streptoavidine as a negative control. HBS-P buffer was used as a running buffer. To anlayze the data for binding, the sensorgram obtained in flow cell for streptoavidine was subtracted from that for MV-H. The data were analyzed by BIAevaluation Software version 4.1.1 (GE Healthcare) with 1:1 binding model.

**Effect of receptors on the 2F4-scFv binding to MV-H. (Competition analysis)**

Competition analysis was carried out using SPR to examine the effect of the receptors on the binding of the 2F4-scFv to MV-H. For this, the 2F4-scFv was mixed with fixed concentration of SLAM (8 μM), nectin-4 (8 μM) or BSA (10 μM), and the solution mixture was injected over the immobilized MV-H. The binding response at equilibrium state, in this case at 70 sec, was plotted against the concentrations of scFv. The binding response was compared between in the presence and absence of SLAM or nectin-4. BSA was used as a control, which does not potentially interact with MV-H.

**Fusion assay using receptor expressing cells**

To test whether 2F4-scFv can inhibit membrane fusion, conventional fusion assay was carried out using receptor expressing Vero cells. One day before experiment, Vero cells stably expressing SLAM or nectin-4 were seeded in the 96-well plate. Next day, when cells reached to 70-80% confluency, the plasmids encoding MV-H, MV-F and GFP were introduced into the receptor expressing Vero cells by transfection. 0.05 μg each per well of the plasmids were mixed with 0.30 μg of PEI max, the DNA mixture was incubated for 15 min at room temperature, subsequently added to the cells. After 5 hours, which enabled cells to express transfected genes, the antibodies, 10 μM of 2F4-scFv as well as 5 μM of MAb 2F4 were added to the assay to examine the effect of antibodies on membrane fusion. The intact 2F4 antibody was used as a control. After 24 hours of the transfection, roughly 18-19 hours of the addition of antibodies, the images were taken and examined the syncytia formation mediated by the binding between MV-H and cellular receptors (Fig. 8).

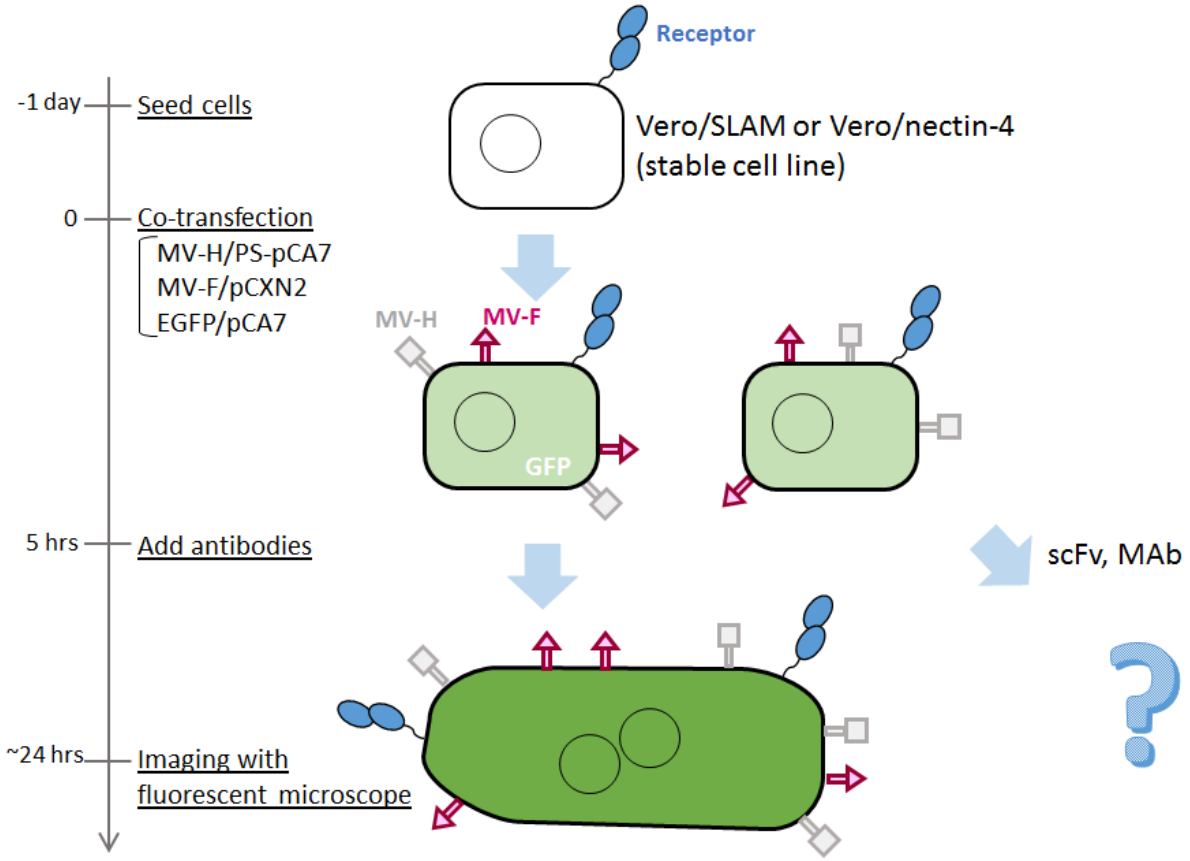


**Figure 8: Schematic diagrams of the conventional fusion assay.**
Vero cells stably expressing SLAM or nectin-4 were used for the assay. Cells were co-transfected with the plasmids expressing viral envelop glycoproteins, MV-H and MV-F, and marker, EGFP. Antibodies were added to the cells 5 hours after transfection, and the cells were observed 24 hours after transfection. When the fusion occurs, the multinucleated huge cells will be observed.

# Results

### Construction of the expression vector of the 2F4-scFv

To address the purpose of this study, the scFv from 2F4 (2F4-scFv) has been constructed by linking $V_H$ and $V_L$ domains with a flexible glycine-serine linker, $(GGGGS)_3$. As I mentioned in the Methods section, the genes encoding $V_H$ and $V_L$ domains of MAb 2F4 were already cloned into pGEM-T vector and the sequences of both domains were determined in our laboratory. Therefore, I began to construct the scFv expression vectors in pET22b with splicing overlap extension PCR method. Both orientations of variable domains were constructed as 2F4-scFv-HL/pET22b and 2F4-scFv-LH/ pET22b. Since $V_H$ and $V_L$ consist of 127 and 105 amino acid residues, the scFv consists of 246 amino acids with a calculated molecular mass of 26,249 Da.

### Expression and purification of recombinant 2F4-scFv Proteins

The recombinant 2F4-scFv was expressed as an inclusion body in *E. coli* regardless of the domain orientations (Fig. 9). Although the expression level of the $V_H$-$V_L$ looked slightly better than that of the $V_L$-$V_H$ , there are no significant differences with each other. Thus, I tried to purify the scFvs from inclusion bodies according to the standard protocol of the refolding with dilution method as described in the method section. Inclusion bodies were diluted into the 400 mM L-Arginine containing refolding buffer to facilitate refolding. L-Arginine is known to function as a solvent to stabilize intermediates in the protein folding. The refolding of the protein was evaluated by size exclusion chromatography (SEC) and the result showed that the peak derived from refolded $V_H$-$V_L$ was observed at around 190 mL, whereas much smaller peaks were obtained for the $V_L$-$V_H$ (Fig. 10). This indicated that the $V_H$-$V_L$ orientation was better

for refolding than the $V_L$-$V_H$ orientation for 2F4-scFv. The yield and refolding efficiency of the scFv with $V_H$-$V_L$ order were calculated to be about 0.5 mg/L-culture and 1.5%, which were relatively low. When I carefully looked at the yield of the every steps of refolding to SEC, most of the protein was lost in the buffer exchange step. Therefore, I sought to find better conditions at this step. In addition, since it seemed difficult to find a good condition for the purification of the 2F4-scFv-LH, the 2F4-scFv-HL was used hereafter as the 2F4-scFv. To examine the buffer condition, I used Tris-HCl, HEPES-NaOH and Sodium phosphate buffers (Fig.11). The concentrated fraction of the refolded protein was buffer-exchanged to each buffer and the same buffer was used as a SEC running buffer. As shown in the Fig. 10, there is no significant improvement for the refolding efficiency in different buffers, 1.7%, 1.1% and 1.6 % for Tris-HCl pH 8.0, HEPES-NaOH pH 8.0 and Sodium phosphate pH 8.0, respectively. Therefore, I kept using Tris-HCl as a basal buffer for purification.

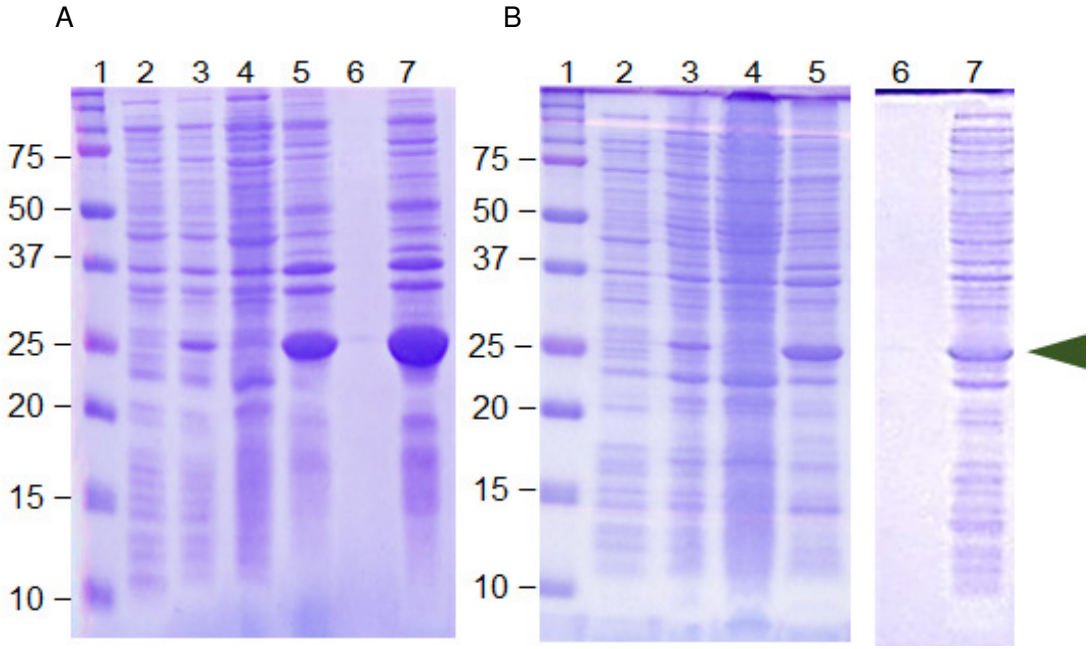


**Figure 9: The SDS-PAGE for the expression and preparation of the inclusion body.** Preparation of the inclusion bodies of 2F4-scFv with $V_H$-$V_L$ (A) or $V_L$-$V_H$ (B). Lane1, Protein size marker; lane 2, before induction; lane 3, after induction; lane 4, supernatant after cell disruption; lane 5, precipitate after cell disruption; lane 6, supernatant after wash of inclusion body; lane 7, precipitate after wash of inclusion body. Arrow indicates the corresponding size of the 2F4-scFv.

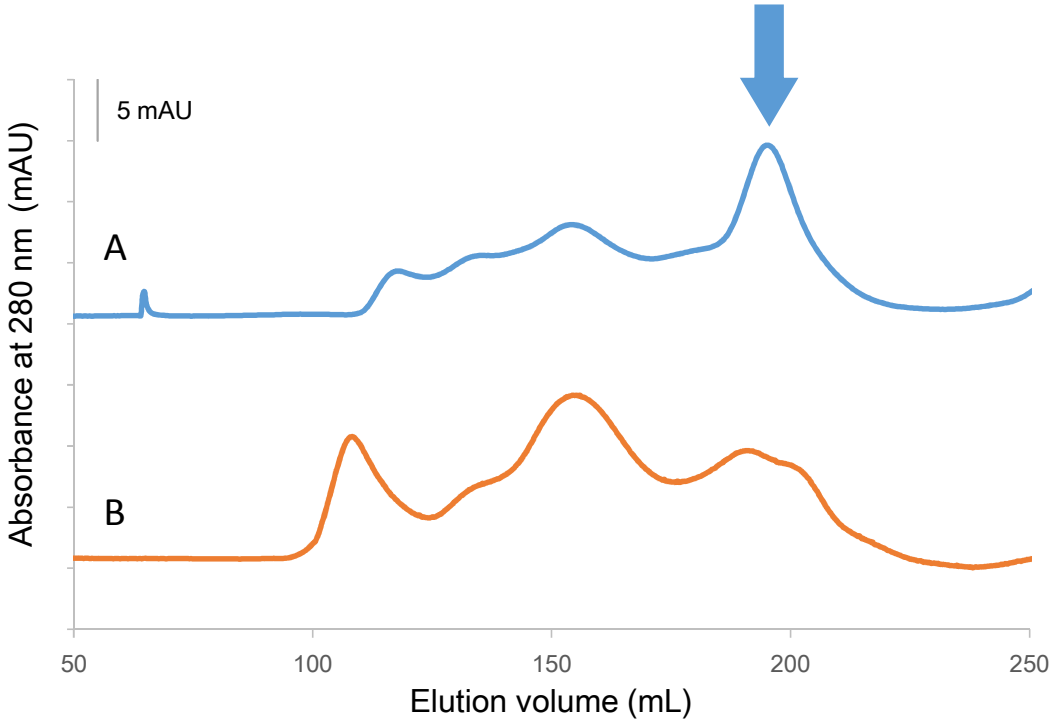


**Figure 10: The chromatograms of the SEC for the 2F4-scFv-HL (A) and 2F4-scFv-LH (B).** The arrow indicates the elution position of the 2F4-scFv.

Next, I tested the L-Arginine condition for buffer exchange to avoid sudden removal of L-Arginine from refolded protein solution. The refolded protein solution, which contains 400 mM L-Arginine, was buffer-exchanged to 50 mM or 100 mM L-Arginine containing SEC running buffer, and applied to SEC using standard running buffer without L-Arginine to remove L-Arginine slowly during SEC. The results showed that the refolding efficiencies were 1.6%, 2.7% and 2.4% when buffer was exchanged to 0 mM, 50 mM and 100 mM L-Arginine containing buffer before SEC (Fig. 12). The slow removal of the L-Arginine slightly improved the refolding efficiency. Since there are no big differences in refolding efficiency between 50 mM and 100 mM L-Arginine (Fig. 12), I decided to use 50 mM L-Arginine condition. It should be noted that the peak eluted around 100 mL, corresponding to void volume become larger when the L-Arginine concentrations were higher (100 mM), suggesting that the amount of soluble aggregates was increased when higher concentration of L-Arginine was removed during SEC.

In addition to these considerations above, the refolding conditions such as buffer pH and L-Arginine concentration for refolding, and concentration conditions, for example decrease the targeted concentration, were also tested although the yield was not dramatically improved as summarized in the Fig. 13. In this way, the yield as well as refolding efficiency for the 2F4-scFv was improved to be about 1.5 mg/L-culture and 4%, respectively.

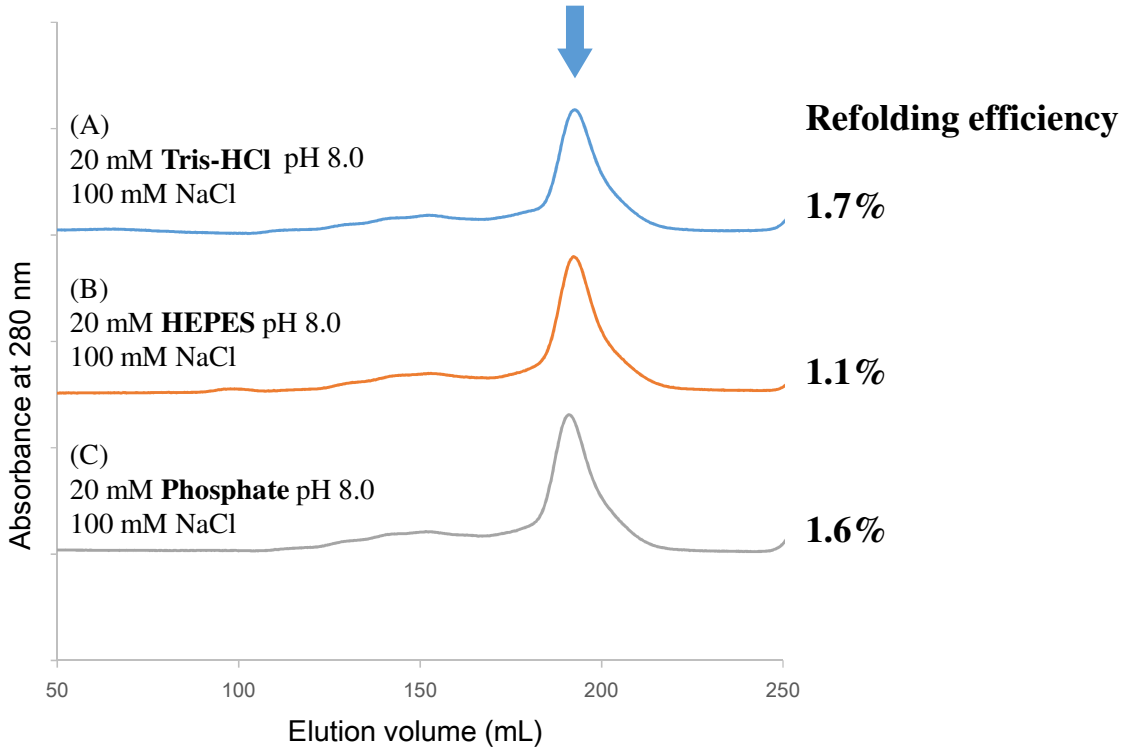


**Figure 11: Examination of the buffer condition for SEC.**
(A) 20 mM Tris-HCl, (B) 20 mM HEPES-NaOH and (C) 20 mM Sodium phosphate were examined for the buffer exchange as well as SEC. The pH for all buffers was 8.0.

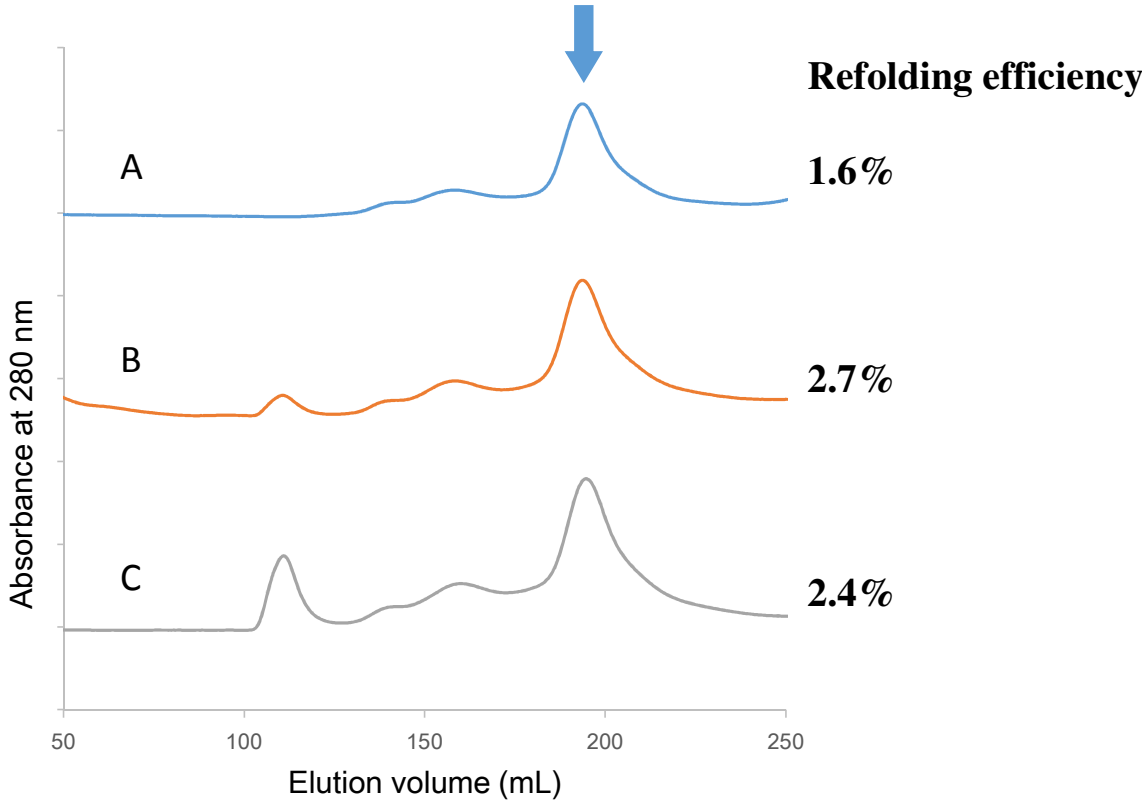


**Figure 12: Examination of the L-Arginine condition before SEC.**
Refolded protein solution was buffer-exchanged to (A) 20 mM Tris-HCl pH 8.0, 100 mM NaCl (SEC running buffer) (B) SEC running buffer containing 50 mM L-Arginine and (C) SEC running buffer containing 100 mM L-Arginine. The SEC was carried out using SEC running buffer.

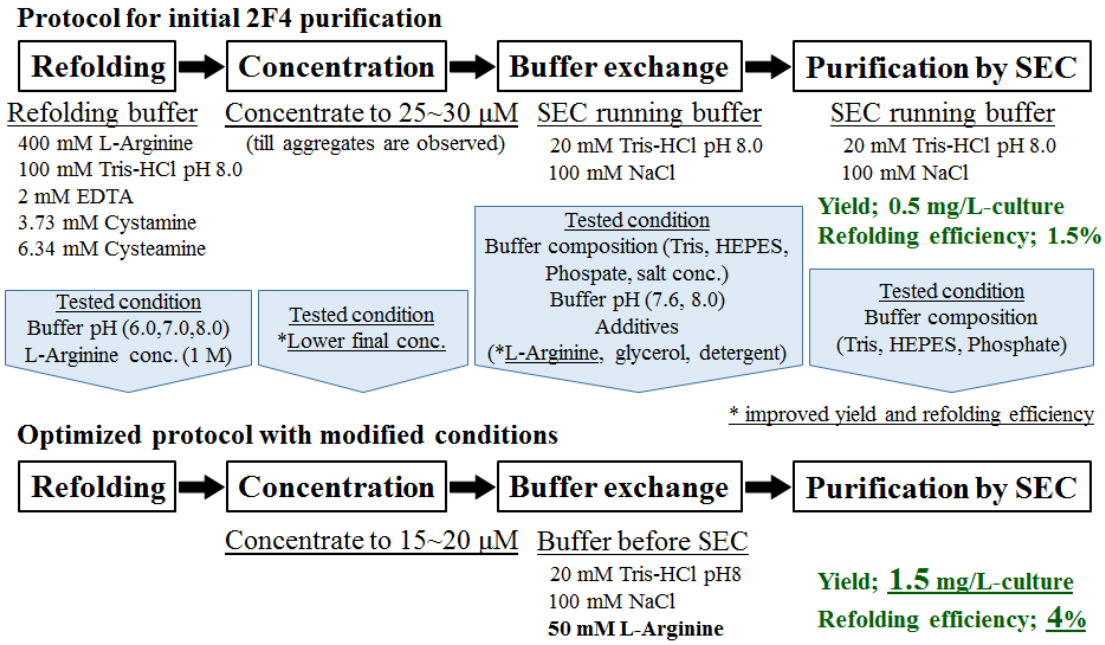


**Figure 13: Summary of the examination for the purification conditions.**

By using the modified condition, the 2F4-scFv was purified and the purity was confirmed by SDS-PAGE (Fig. 14). When compared with the molecular size marker indicated above the chromatogram, the molecular mass was estimated to be 25 kDa based on the elution position of the protein containing fraction (Fig. 14A), which is in good agreement with the molecular size of monomer scFv, 26 kDa calculated by the amino acid sequence. The purity of the recombinant scFv was >95% confirmed by SDS-PAGE (Fig. 14B), that is enough quality for the later experiments including SPR analysis.

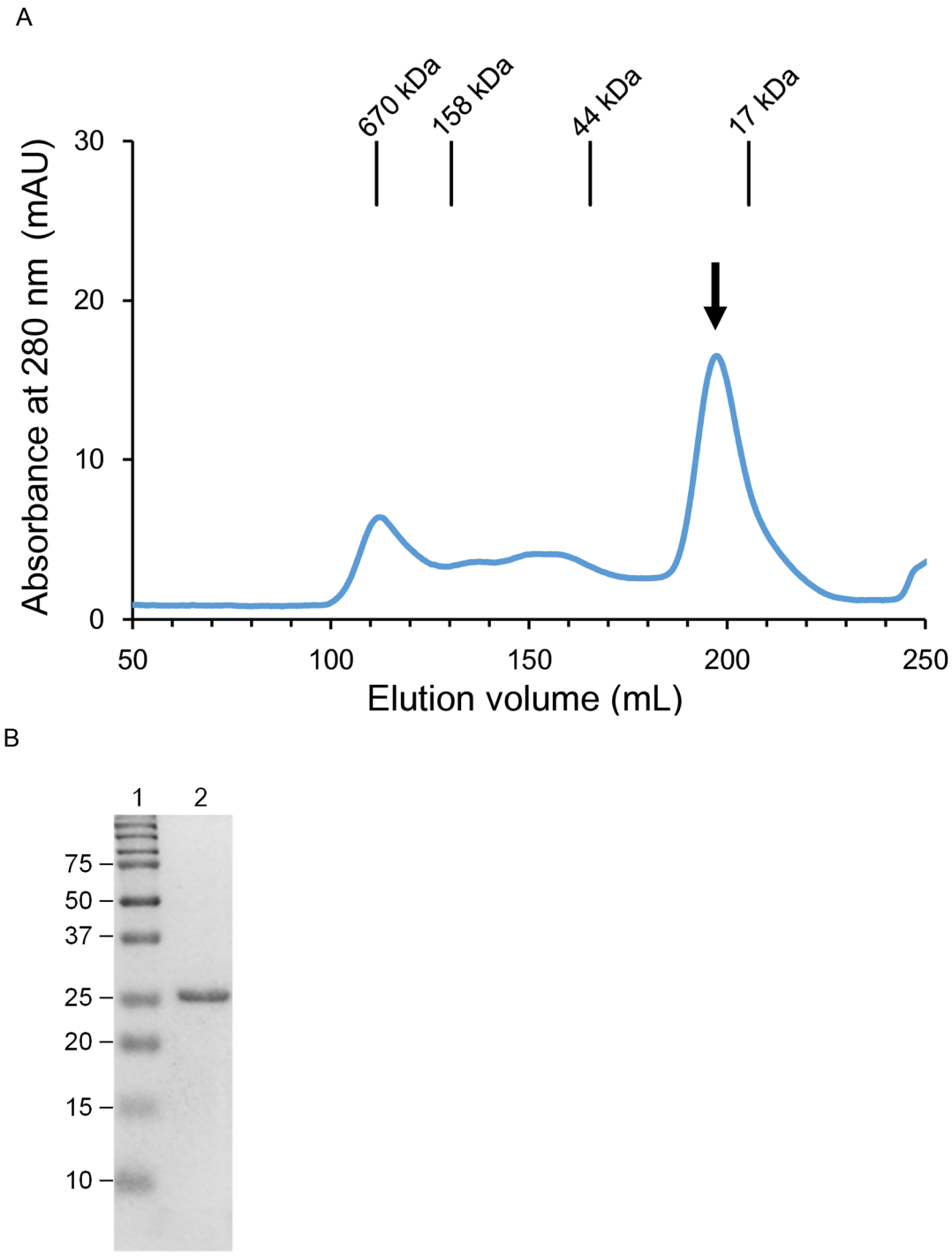


**Figure 14: Purification of the recombinant 2F4-scFv protein.**
(A) Chromatogram of the size exclusion chromatography. The arrow indicates the elution position of the 2F4-scFv. The elution position of the molecular size marker was indicated above the chromatogram. (B) SDS-PAGE with Coomassie staining. Lane1, molecular size marker; lane 2, 2 µg of purified 2F4-scFv.

**Binding kinetics of 2F4-scFv, 2F4 Fab, SLAM and nectin-4 to the MV-H.**

To examine whether the 2F4-scFv exhibits functional binding affinity to MV-H protein, I performed kinetic analyses using SPR. For this, MV-H from Edmonston strain (MV-H(Ed)) was used as a ligand since it can be obtained with better yield than the MV-H from wild type (IC-B) strain. The SLAM and nectin-4 binding sites are conserved within both strains, and intact 2F4 can inhibit the binding of SLAM with similar manner.

The 2F4-scFv showed binding affinity of $K_D$ = 5.0 μM to MV-H (Fig. 15A, Table 3). The association and dissociation kinetic constants, $k_a$ and $k_d$, for 2F4-scFv were $3.6x10^3$ $M^{-1}$ $s^{-1}$ and $18x10^{-3}$ $s^{-1}$, respectively (Table 3). To reveal whether the scFv fragmentation affects the binding affinity to MV-H, the kinetic analysis for 2F4-Fab was carried out. For this, the 2F4-Fab was prepared by digesting MAb 2F4 with papain. The $K_D$ value of 2F4-Fab was determined to 0.018 μM, approximately 280 times smaller $K_D$ value than 2F4-scFv, indicating a higher affinity than the 2F4-scFv. (Fig. 15B, Table 3). The difference in $K_D$ value between the scFv and Fab was due to both the association and dissociation rate constants differences, roughly 50 times larger $k_a$ and 6 times smaller $k_d$. To compare with the affinity between 2F4-scFv and cellular receptors, the kinetic analysis was performed for both SLAM and nectin-4, the kinetic parameters of the binding between SLAM or nectin-4 and MV-H were also determined and the results showed the binding affinity of $K_D$ = 0.24 μM and $K_D$ = 0.67 μM for SLAM and nectin-4, respectively (Fig. 15C and D, Table 3). The $K_D$ value of scFv was approximately 20 and 7 times larger than those of the SLAM and nectin-4. When the association and dissociation kinetic constants ($k_a$ and $k_d$) to MV-H were compared between scFv and SLAM, the $k_a$ value of scFv was roughly 4 times smaller than that of SLAM, whereas

the $k_d$ value was approximately 5 times larger (Table 3), indicating that the differences of both $k_a$ and $k_d$ values affect the differences of the binding affinities between scFv and SLAM. On the other hand, the $k_a$ value of nectin-4 was roughly 6 times larger than that of scFv, while the $k_d$ values for both scFv and nectin-4 were comparable, suggesting that the faster association kinetics of nectin-4 may be responsible for its higher affinity to MV-H than that of scFv.

We also analyzed the equilibrium binding of those proteins to MV-H using sensorgrams shown in Figure 15. The responses at 70 sec, where response reached almost equilibrium binding, were plotted against the analyte concentrations, and the plots were fitted directly to the non-linear 1:1 binding model. In this way, the $K_D$ values for scFv, Fab, SLAM and nectin-4 were estimated to be 5.9 μM, 0.070 μM , 0.72 μM, and 1.0 μM, respectively (Fig. 16), which were comparable to those calculated by the kinetic analysis (Fig. 15, Table 3).

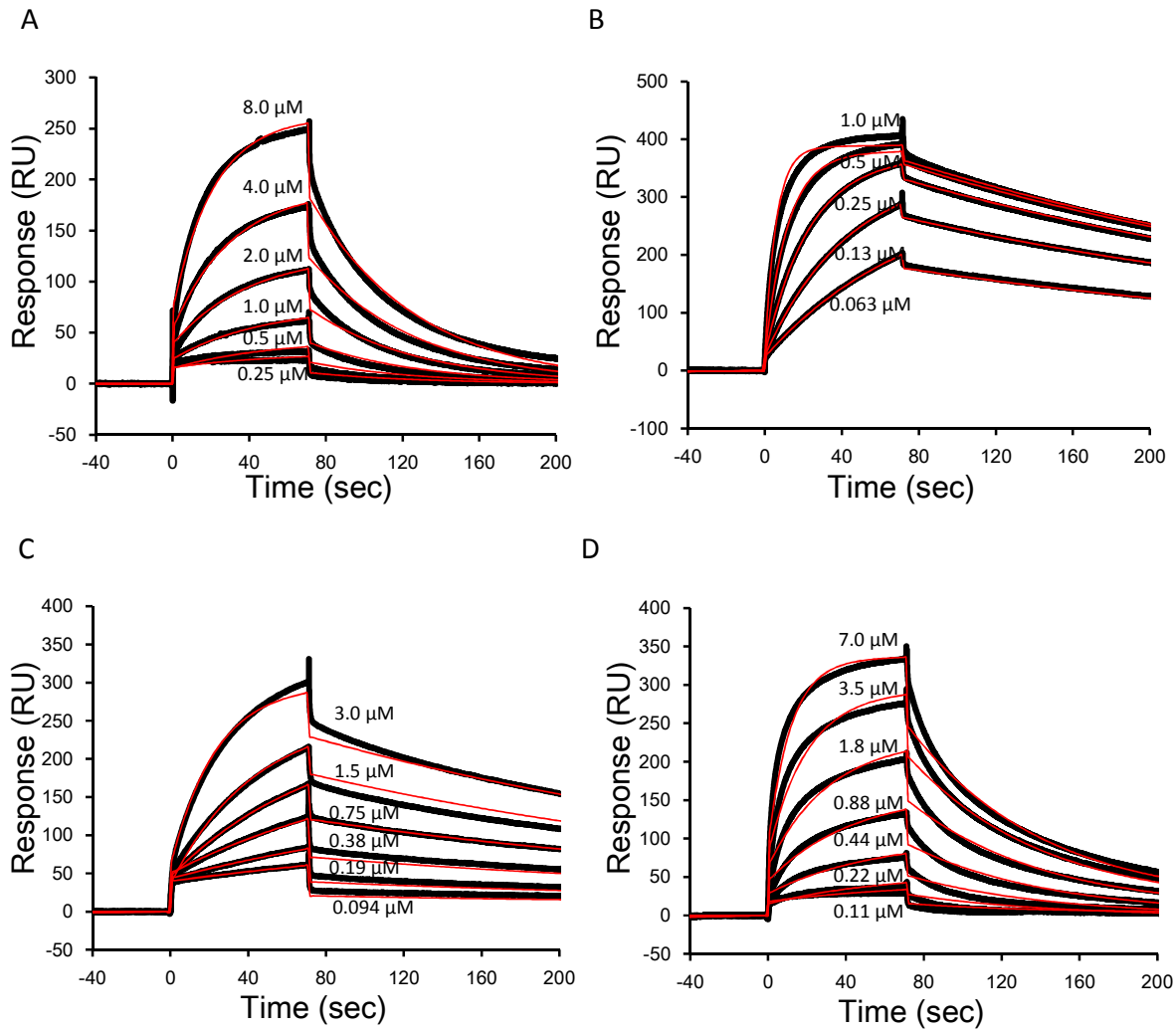


**Figure 15: Kinetic analyses of 2F4-scFv, 2F4 Fab, SLAM or nectin-4 with MV-H.**
(A) 2F4-scFv, (B) 2F4 Fab, (C) SLAM and (D) nectin-4 at different concentrations were injected over the immobilized MV-H and the representative sensorgrams are shown. Response curves were fitted by the simple 1:1 Langmuir binding model. The black line indicates raw data of the response curves, whereas the thin red line indicates the fitting curves. The kinetic parameters were summarized in Table 3.

**Table 3: Kinetic parameters of antibody or cellular receptors to the MV-H.**

| Analyte | Ligand | $K_D$ (μM) | $k_a$ ($M^{-1}$ $s^{-1}$) | $k_d$ ($s^{-1}$) |
|---|---|---|---|---|
| 2F4-scFv | MV-H (Ed) | 5.0 | $3.6x10^3$ | $18x10^{-3}$ |
| 2F4-Fab | MV-H (Ed) | 0.018 | $1.7x10^5$ | $3.0x10^{-3}$ |
| SLAM | MV-H (Ed) | 0.24 | $1.5x10^4$ | $3.3x10^{-3}$ |
| nectin-4 | MV-H (Ed) | 0.67 | $2.1\times10^4$ | $14\times10^{-3}$ |

The analyte was injected over the immobilized MV-H protein. The kinetic parameters of binding were calculated by the fitting with the simple 1:1 Langmuir binding model. The equilibrium dissociation constant, $K_D$ values were calculated with the equation $K_D = k_d/k_a$ (*eq 5*).

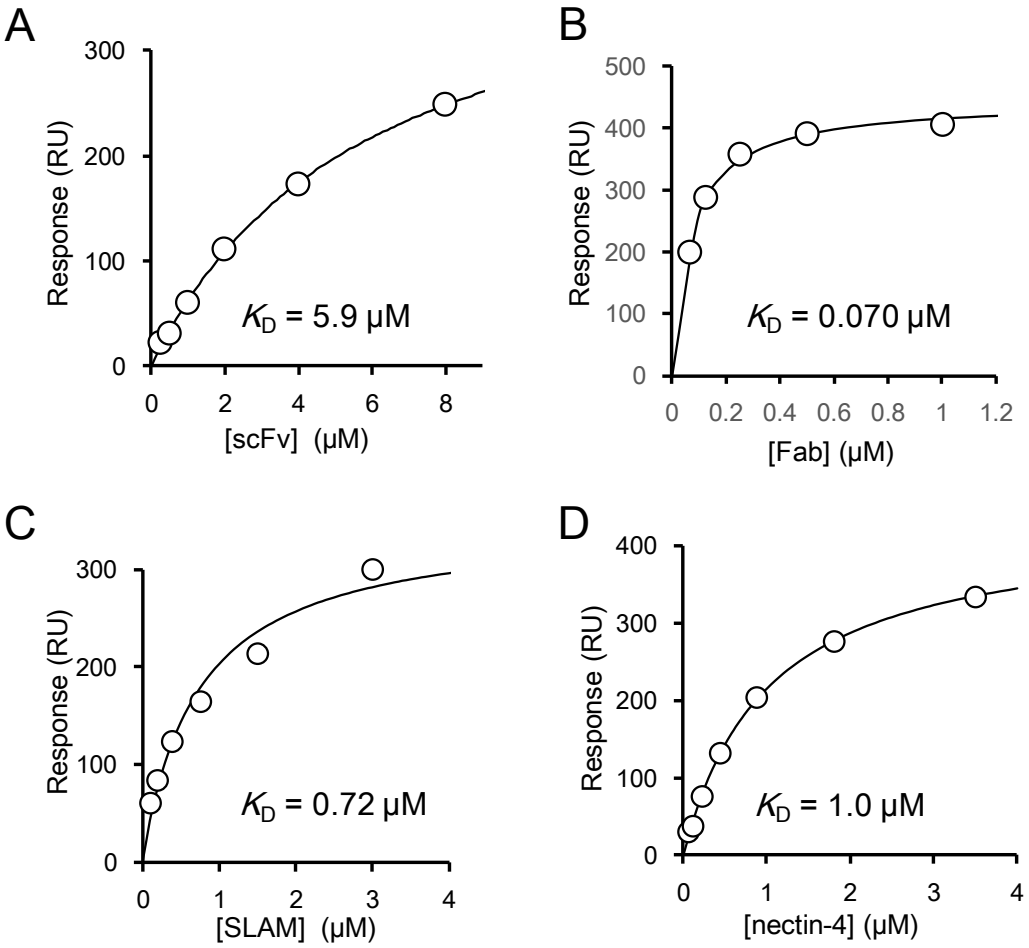


**Figure 16: The equilibrium binding of scFv (A), SLAM (B), and nectin-4 (C) to MV-H.** The responses of equilibrium binding at 70 sec in Figure 14 were plotted against each analyte concentration. The solid lines indicate direct nonlinear fits of the 1:1 binding model.

**Effect of receptors on the 2F4-scFv binding to MV-H**

Next, we examined whether the 2F4-scFv can directly inhibit the binding of cellular receptors, SLAM and nectin-4, to the MV-H although the intact antibody is known to inhibit the fusion of the recombinant MVs to the cells (42). As shown above, however, both SLAM and nectin-4 bind to MV-H at sub micromolar order of $K_D$, which is roughly 10 times higher affinity than that of the 2F4-scFv (Fig. 15, Table 3). We therefore analyzed whether the binding response of the scFv is affected by the saturated concentration of SLAM or nectin-4. The scFv was mixed with SLAM or nectin-4 and the mixture was injected over the immobilized MV-H and the binding responses at equilibrium state were compared with and without each receptor. The results showed that the binding responses of scFv rarely or did not increase in the presence of 8 μM SLAM (Fig. 17A, filled circles) or 8 μM nectin-4 (Fig. 17B, filled circles). When the response of the scFv alone was subtracted from that of the scFv with SLAM or nectin-4, the resultant values decreased as the concentrations of scFv increased (Fig. 17 A and B, crosses), indicating that the binding of the scFv to MV-H was affected by the presence of both SLAM and nectin-4. On the other hand, the binding responses were increased in the presence of 10 μM BSA (Fig. 17C, filled circles) with similar manner without BSA (Fig. 17C, open circles), indicating that the binding of the scFv to MV-H did not altered by BSA at all. This suggest that the binding of the 2F4-scFv to MV-H was specifically inhibited by both SLAM and nectin-4 in a competitive manner. Conversely, the 2F4-scFv may be able to inhibit the binding of cellular receptors to MV-H when the scFv abundantly exists. Taken together, the inhibition of the binding of cellular receptors to MV-H is the competitive and specific for the 2F4-scFv.

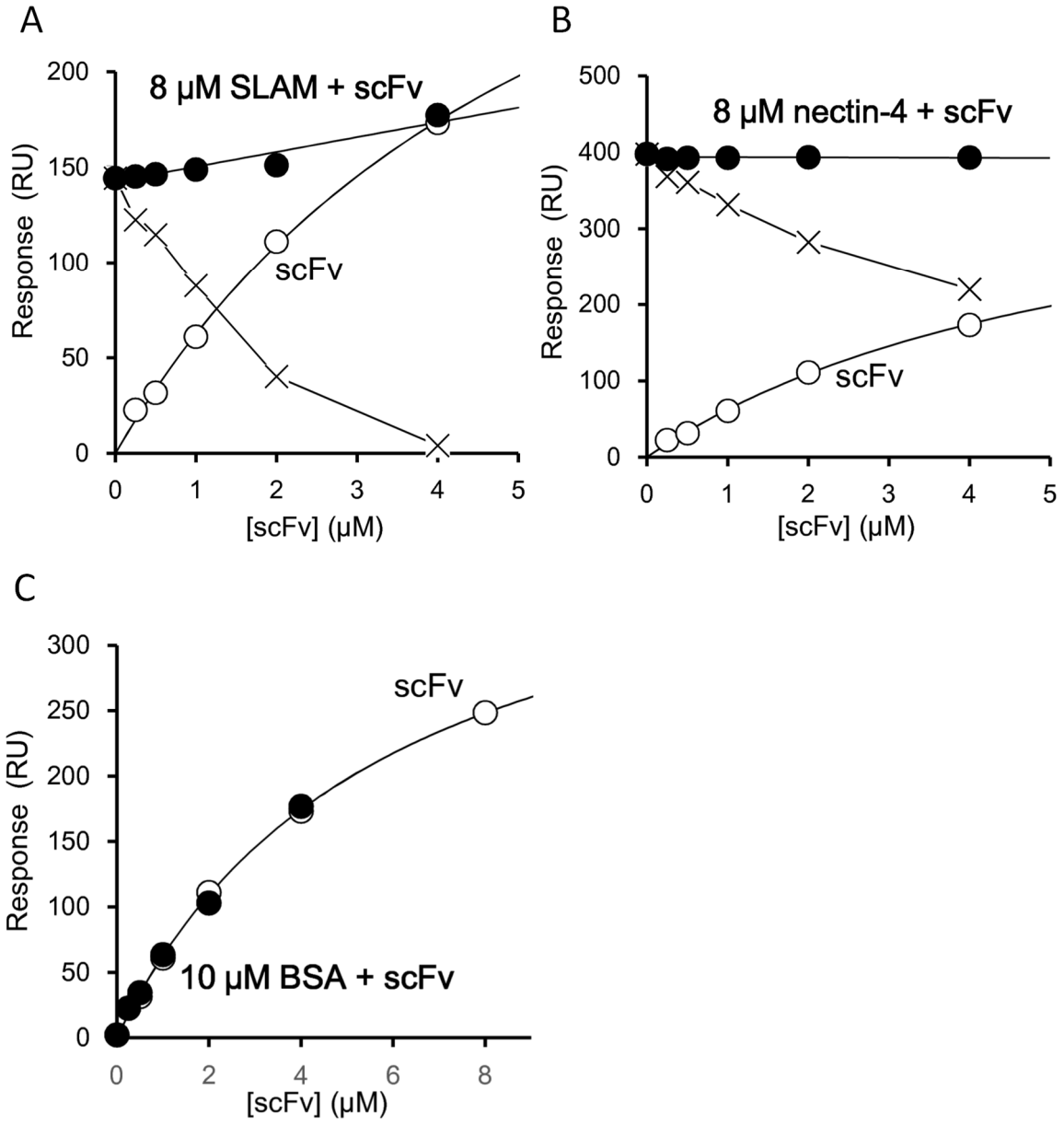


**Figure 17: The effect of SLAM or nectin-4 on the binding of the 2F4-scFv to MV-H**.
(A) Binding of 2F4-scFv in the absence (open circles) or presence (filled circles) of 8 μM hSLAM. The differences of the scFv binding with or without SLAM were also plotted (crosses). (B) Binding of 2F4-scFv in the absence (open circles) or presence (filled circles) of 8 μM nectin-4. The differences of the scFv binding with or without nectin-4 were also plotted (crosses). (C) Binding of 2F4-scFv in the absence (open circles) or presence (filled circles) of 10 μM BSA.

**Effect of the 2F4-scFv on the membrane fusion**

We examined the effect of the 2F4-scFv on the membrane fusion assessed by the fusion assay. We expected that the scFv can inhibit membrane fusion since the binding of scFv to MV-H were revealed to be competitive with the binding of cellular receptors (Fig. 17) though its affinity to MV-H is low. To determine whether the 2F4-scFv can inhibit membrane fusion, conventional fusion assay was performed using Vero cells stably expressing SLAM or nectin-4. The Vero cells were co-transfected with the plasmids expressing MV-H, MV-F and EGFP, and the syncytia formation was observed by fluorescent microscopy. We used 10 μM of scFv because of low affinity to MV-H. As expected, multinucleated huge cells were observed in both SLAM- and nectin-4-expressing Vero cells (Fig. 18, left panel). Notably, the syncytia formation was not observed when the cells were not transfected with either MV-H or MV-F expressing plasmid, indicating that the cell-cell fusion observed in this assay was mediated only by the involvement of MV-H, -F and cellular receptors. On the other hand, the syncytia formation was not observed when 10 μM of 2F4-scFv as well as 5 μM of MAb 2F4 were added to the assay (Fig. 18, middle and right panels). The result revealed that the 2F4-scFv was able to inhibit syncytia formation (membrane fusion) mediated by the interaction between MV-H and cellular receptors.

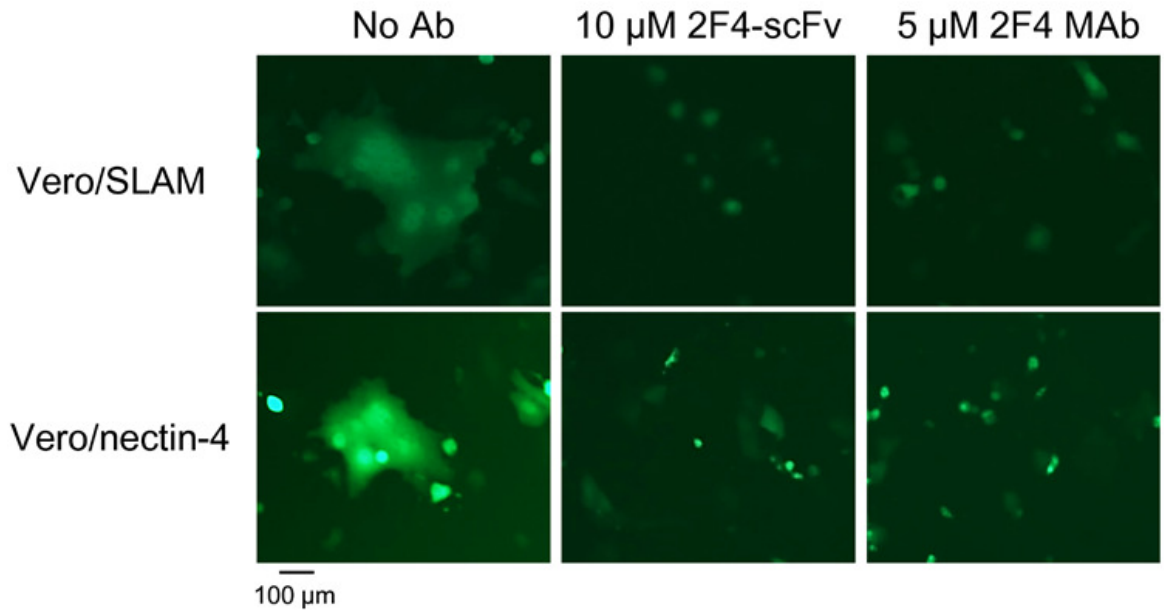


**Figure 18: The 2F4-scFv can inhibit the membrane fusion.**
The fusion assay was conducted by observing syncytia formation in the presence or absence of 2F4-scFv as well as in the presence of intact 2F4 as a control. The representative images were shown for the untreated and antibodies treated cells.

# Discussion

Here, I focused on the 2F4-scFvs due to the beneficial effect of scFv format than intact antibodies, it is smaller in size with retaining antigen binding specificity, has less possibility of developing immunogenicity, easy to be modified via protein engineering approach, and useful for structural study (55). However, affinity can be sometimes reduced when fragmented. Indeed, the 2F4-scFv has lower affinity to MV-H, roughly 300 times, than Fab form. The scFv can be administered into body by intracerebral injection, intranasal administration and viral transduction and thereby delivered throughout the brain (56-58), which is subjected to study to reduce the fatal effect from subacute sclerosing panencephalitis (SSPE).

For the preparation of 2F4-scFv in purified form, I observed $V_H$-$V_L$ orientation was refolded and eluted by size exclusion chromatography in soluble monomer form, whereas $V_L$-$V_H$ orientation was not refolded properly under the tested condition (Fig. 10). It may be possible that the $V_H$ domain is more stable in folding than the $V_L$ domain, thus $V_H$-$V_L$ orientation is favorable for (re)folding in the case of the 2F4. It has been shown for scFv antibody against c-Met, a receptor of hepatocyte growth factor/scattering Factor, antibody with the better productivity of $V_H$-$V_L$ scFv (59). In the case of the scFv antibody against PSMA, prostate-specific membrane antigen, $V_H$-$V_L$ form showed the highest expression and antigen binding, while $V_L$-$V_H$ form exhibited the best cytotoxicity (60). Thus, the $V_H$ and $V_L$ orientation should be considered to obtain a good fragmented antibody in terms of the productivity and the quality.

Initially, the yield and refolding efficiency of 2F4-scFv were relatively low, about 0.5 mg/L-culture and 1.5%, respectively. In the efforts for the examination of the purification condition, such as consideration of refolding, concentration, and buffer

exchange conditions, I could establish the better preparation method for 2F4-scFv with slight improvement of the yield and refolding efficiency to about 1.5 mg/L-culture and 4%, respectively. I am planning to do crystallization of the complex between scFv and MV-H to identify the antigenic epitopes, however the current yield is not sufficient for the study. Therefore, further modification for refolding, such as the ratio of oxidizing and reducing agents which is sometimes critical for the refolding of proteins, must be tested. The binding affinity of 2F4 is largely different between scFv and Fab forms (more than 250 times difference), suggesting that the rigidity of the molecule may be different with each other, indirectly account for the instability of the scFv form. Therefore, the mutagenesis in the framework region of the antibody may be alternative approach to improve stability without any change in the antigen specificity. The length and sequence of the linker connecting $V_H$ and $V_L$ domains also need to be considered (50).

I have determined the binding properties of 2F4-scFv to MV-H by SPR (Fig. 15, Table 3). The results revealed that the 2F4-scFv can bind with lower affinity than its Fab fragment, produced from the same MAb and both cellular receptors, SLAM and nectin-4. Several researches have mentioned the reduced antigen binding affinity of scFv that is one of the possible drawback of scFv (45,61-63). One of the possible reasons is that the interface of both variable domains become loosen, resulted from the fragmentation, thereby the conformation of the CDRs may slightly altered. Another possibility is that the loop of the linker may interfere the configuration of the CDR loops slightly. Indeed, the binding kinetics of scFv is quite different from that of Fab although the specificity to antigen binding retained. Thus, the central loop for antigen recognition, CDR3 located at the center of the antigen recognition thumbs, did not

affected seriously, but association and dissociation rate constant severely altered following fragmentation probably because of the alteration of the other CDRs, CDR1 or CDR2 located relatively outside of the antigen recognition site of the antibody. To date, several techniques are available to generate higher affinity mutants of scFv, such as site directed mutagenesis and phage-based random mutagenesis (45,48,64,65). So in the future, I would like to improve the affinity of 2F4-scFv by applying these techniques.

Although the affinity of the 2F4-scFv is lower than cellular receptors (Table 3), I examined whether the scFv can inhibit the interaction of MV-H to cellular receptors, SLAM or nectin-4, and membrane fusion mediated by the interaction of MV-H to cellular receptors. The competitive analysis using SPR as well as the conventional fusion assay clearly indicated that the 2F4-scFv possesses inhibitory activity of the binding of both SLAM and nectin-4 to MV-H, thereby to inhibit the membrane fusion, although the sufficient concentration of the scFv to the receptors was required. It is because the scFv and cellular receptors can interact with MV-H in a competitive manner. Indeed, we used 10 μM of scFv for the conventional fusion assay, which was roughly $10^4$ – $10^5$ times higher concentration than the intact antibody used for the previous virus infection assay (42) though the assay strategies differ with each other. For the use of the scFv in the actual therapy, therefore the improvement of the binding affinity to MV-H is urgently needed as mentioned above. The kinetic binding analysis shows that the both $k_a$ and $k_d$ were likely altered as compared to those of Fab after constructing scFv (Fig. 15, Table 3). Thus, the mutations that improve both association and dissociation rate constants will be required. Nevertheless, the results in this study are consistent with the previous virus neutralizing assay conducted by using intact 2F4 MAb (42), suggesting that there is no significant alteration by following fragmentation of the antibody, and the

2F4 fragment form may be sufficient for the inhibition of the membrane fusion. It should be emphasized that our SPR analysis directly showed the competitive binding mode of the scFv and cellular receptors to MV-H. Moreover, these results clearly account for the hypothesis that the 2F4 uses the receptor binding site as a neutralizing epitope. To identify the epitope of 2F4-scFv, I am planning to conduct comprehensive mutagenesis studies on MV-H targeted to the receptor binding sites and binding analysis, and crystallographic studies for the antibody fragments with MV-H proteins. These will provide the future aspect of the 2F4-scFv in effective vaccine development and anti-viral drug for MV infection.

And for SSPE, I need to study about the blood brain crossing ability of 2F4-scFv to deliver it directly into the brain, then I can address this future for SSPE. Ultimately, I would like to make a construct, intruducing antigenic epitope to the plasmid vector for scFv to get synergistic effect for MV infection followed by checking for antibody production, as well as for cytokine, ultimately for effectiveness about the construct.

# Conclusion

In this study, I successfully constructed the 2F4-scFvs from MAb 2F4, found that the 2F4-scFv with $V_H$-$V_L$ orientation is better for refolding, and established the preparation method for 2F4-scFv. The kinetic analyses using SPR suggested that the 2F4-scFv specifically binds to MV-H head domain at μM level of $K_D$ value, which is lower affinity than those of Fab and cellular receptors, SLAM and nectin-4. Further competitive binding analysis demonstrated that the 2F4-scFv directly inhibits the binding of MV-H to SLAM and nectin-4. Finally, fusion assay using receptor expressing cells revealed that the 2F4-scFv show sufficient inhibitory effect on the membrane fusion mediated by the binding of MV-H to cellular receptors. Collectively, the findings in this study directly account for the hypothesis that the 2F4 uses the receptor binding sites as neutralizing epitopes. 2F4-scFv can be excellent candidate and tool for understanding the inhibitory mechanism of virus entry to cells via MV-H, leading to design an effective vaccine and anti-viral drug for MV. It will be also applicable for the development of viral and nonviral vectors for gene delivery for antigenic epitope to stimulate antibody production by immune cell and ultimately provide synergistic effect against MV-infection resulting from prophylactic and/or therapeutic purpose. In order to address this for the future, the epitope of 2F4 needs to be determined by mutagenesis and structural studies. Furthermore the binding affinity of the 2F4-scFv to MV-H also needs to be improved with both rational and/or random designs for *in vivo* effectiveness of the scFv by using mouse model. Then 2F4-scFv can be effective therapy for measles infection to human who are unable to produce antibody by vaccination.

# References


1. Rota, P., Brown, K., Mankertz, A., Santibanez, S., Shulga, S., Muller, C., Hubschen, J., Siqueira, M., Beirnes, J., Ahmed, H., Triki, H., Al-Busaidy, S., Dosseh, A., Byabamazima, C., Smit, S., Akoua-Koffi, C., Bwogi, J., Bukenya, H., Wairagkar, N., Ramamurty, N., Incomserb, P., Pattamadilok, S., Jee, Y., Lim, W., Xu, W., Komase, K., Takeda, M., Tran, T., Castillo-Solorzano, C., Chenoweth, P., Brown, D., Mulders, M., Bellini, W., and Featherstone, D. (2011) Global Distribution of Measles Genotypes and Measles Molecular Epidemiology. *Journal of Infectious Diseases* **204**, S514-S523
2. Rota, P., Brown, K., Hubschen, J., Muller, C., Icenogle, J., Chen, M., Bankamp, B., Kessler, J., Brown, D., Bellini, W., and Featherstone, D. (2011) Improving Global Virologic Surveillance for Measles and Rubella. *Journal of Infectious Diseases* **204**, S506-S513
3. Moss, W., and Griffin, D. (2012) Measles. *Lancet* **379**, 153-164
4. Reuter, D., and Schneider-Schaulies, J. (2010) Measles virus infection of the CNS: human disease, animal models, and approaches to therapy. *Medical Microbiology and Immunology* **199**, 261-271
5. Yanagi, Y., Takeda, M., and Ohno, S. (2006) Measles virus: cellular receptors, tropism and pathogenesis. *Journal of General Virology* **87**, 2767-2779
6. Tatsuo, H., Ono, N., Tanaka, K., and Yanagi, Y. (2000) SLAM (CDw150) is a cellular receptor for measles virus. *Nature* **406**, 893-897
7. Noyce, R., Bondre, D., Ha, M., Lin, L., Sisson, G., Tsao, M., and Richardson, C. (2011) Tumor Cell Marker PVRL4 (Nectin 4) Is an Epithelial Cell Receptor for Measles Virus. *Plos Pathogens* **7**

8. Muhlebach, M., Mateo, M., Sinn, P., Prufer, S., Uhlig, K., Leonard, V., Navaratnarajah, C., Frenzke, M., Wong, X., Sawatsky, B., Ramachandran, S., McCray, P., Cichutek, K., von Messling, V., Lopez, M., and Cattaneo, R. (2011) Adherens junction protein nectin-4 is the epithelial receptor for measles virus. *Nature* **480**, 530-U153

9. DORIG, R., MARCIL, A., CHOPRA, A., and RICHARDSON, C. (1993) THE HUMAN CD46 MOLECULE IS A RECEPTOR FOR MEASLES-VIRUS (EDMONSTON STRAIN). *Cell* **75**, 295-305

10. NANICHE, D., VARIORKRISHNAN, G., CERVONI, F., WILD, T., ROSSI, B., RABOURDINCOMBE, C., and GERLIER, D. (1993) HUMAN MEMBRANE COFACTOR PROTEIN (CD46) ACTS AS A CELLULAR RECEPTOR FOR MEASLES-VIRUS. *Journal of Virology* **67**, 6025-6032

11. Yanagi, Y., Takeda, M., Ohno, S., Hashiguchi, T., Griffin, D., and Oldstone, M. (2009) Measles Virus Receptors. *Measles: History and Basic Biology* **329**, 13-30

12. Dhiman, N., Jacobson, R., and Poland, G. (2004) Measles virus receptors: SLAM and CD46. *Reviews in Medical Virology* **14**, 217-229

13. Schwartzberg, P., Mueller, K., Qi, H., and Cannons, J. (2009) SLAM receptors and SAP influence lymphocyte interactions, development and function. *Nature Reviews Immunology* **9**, 39-46

14. Kiel, M., Yilmaz, O., Iwashita, T., Terhorst, C., and Morrison, S. (2005) SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* **121**, 1109-1121

15. Mavaddat, N., Mason, D., Atkinson, P., Evans, E., Gilbert, R., Stuart, D., Fennelly, J., Barclay, A., Davis, S., and Brown, M. (2000) Signaling

lymphocytic activation molecule (CDw150) is homophilic but self-associates with very low affinity. *Journal of Biological Chemistry* **275**, 28100-28109

16. COCKS, B., CHANG, C., CARBALLIDO, J., YSSEL, H., DEVRIES, J., and AVERSA, G. (1995) A NOVEL RECEPTOR INVOLVED IN T-CELL ACTIVATION. *Nature* **376**, 260-263

17. Hashiguchi, T., Ose, T., Kubota, M., Maita, N., Kamishikiryo, J., Maenaka, K., and Yanagi, Y. (2011) Structure of the measles virus hemagglutinin bound to its cellular receptor SLAM. *Nature Structural & Molecular Biology* **18**, 135-U191

18. Reymond, N., Fabre, S., Lecocq, E., Adelaide, J., Dubreuil, P., and Lopez, M. (2001) Nectin4/PRR4, a new afadin-associated member of the nectin family that trans-interacts with Nectin1/PRR1 through V domain interaction. *Journal of Biological Chemistry* **276**, 43205-43215

19. Brancati, F., Fortugno, P., Bottillo, I., Lopez, M., Josselin, E., Boudghene-Stambouli, O., Agolini, E., Bernardini, L., Bellacchio, E., Iannicelli, M., Rossi, A., Dib-Lachachi, A., Stuppia, L., Palka, G., Mundlos, S., Stricker, S., Kornak, U., Zambruno, G., and Dallapiccola, B. (2010) Mutations in PVRL4, Encoding Cell Adhesion Molecule Nectin-4, Cause Ectodermal Dysplasia-Syndactyly Syndrome. *American Journal of Human Genetics* **87**, 265-273

20. Takai, Y., Miyoshi, J., Ikeda, W., and Ogita, H. (2008) Nectins and nectin-like molecules: roles in contact inhibition of cell movement and proliferation. *Nature Reviews Molecular Cell Biology* **9**, 603-615

21. Takai, Y., and Nakanishi, H. (2003) Nectin and afadin: novel organizers of intercellular junctions. *Journal of Cell Science* **116**, 17-27

22. Ogita, H., and Takai, Y. (2006) Nectins and nectin-like molecules: Roles in cell adhesion, polarization, movement, and proliferation. *Iubmb Life* **58**, 334-343

23. Manchester, M., Gairin, J., Patterson, J., Alvarez, J., Liszewski, M., Eto, D., Atkinson, J., and Oldstone, M. (1997) Measles virus recognizes its receptor, CD46, via two distinct binding domains within SCR1-2. *Virology* **233**, 174-184

24. HU, A., SHESHBERADARAN, H., NORRBY, E., and KOVAMEES, J. (1993) MOLECULAR CHARACTERIZATION OF EPITOPES ON THE MEASLES-VIRUS HEMAGGLUTININ PROTEIN. *Virology* **192**, 351-354

25. Fournier, P., Brons, N., Berbers, G., Wiesmuller, K., Fleckenstein, B., Schneider, F., Jung, G., and Muller, C. (1997) Antibodies to a new linear site at the topographical or functional interface between the haemagglutinin and fusion proteins protect against measles encephalitis. *Journal of General Virology* **78**, 1295-1302

26. Ertl, O., Wenz, D., Bouche, F., Berbers, G., and Muller, C. (2003) Immunodominant domains of the Measles virus hemagglutinin protein eliciting a neutralizing human B cell response. *Archives of Virology* **148**, 2195-2206

27. Bouche, F., Ertl, O., and Muller, C. (2002) Neutralizing B cell response in measles. *Viral Immunology* **15**, 451-471

28. Hashiguchi, T., Maenaka, K., and Yanagi, Y. (2011) Measles virus hemagglutinin: structural insights into cell entry and measles vaccine. *Frontiers in Microbiology* **2**

29. Chothia, C., and Jones, E. (1997) The molecular structure of cell adhesion molecules. *Annual Review of Biochemistry* **66**, 823-862

30. Cao, E., Ramagopal, U., Fedorov, A., Fedorov, E., Yan, Q., Lary, J., Cole, J.,

Nathenson, S., and Almo, S. (2006) NTB-A receptor crystal structure: Insights into homophilic interactions in the signaling lymphocytic activation molecule receptor family. *Immunity* **25**, 559-570

31. Yuan, P., Leser, G., Demeler, B., Lamb, R., and Jardetzky, T. (2008) Domain architecture and oligomerization properties of the paramyxovirus PIV 5 hemagglutinin-neuraminidase (HN) protein. *Virology* **378**, 282-291

32. Paal, T., Brindley, M., St Clair, C., Prussia, A., Gaus, D., Krumm, S., Snyder, J., and Plemper, R. (2009) Probing the Spatial Organization of Measles Virus Fusion Complexes. *Journal of Virology* **83**, 10480-10493

33. de Swart, R., Yuksel, S., and Osterhaus, A. (2005) Relative contributions of measles virus hemagglutinin- and fusion protein-specific serum antibodies to virus neutralization. *Journal of Virology* **79**, 11547-11551

34. de Swart, R., Yuksel, S., Langerijs, C., Muller, C., and Osterhaus, A. (2009) Depletion of measles virus glycoproteln-specific antibodies from human sera reveals genotype-specific neutralizing antibodies. *Journal of General Virology* **90**, 2982-2989

35. Bellini, W., and Rota, P. (2011) Biological feasibility of measles eradication. *Virus Research* **162**, 72-79

36. Finsterbusch, T., Wolbert, A., Deitemeier, I., Meyer, K., Mosquera, M., Mankertz, A., and Santibanez, S. (2009) Measles viruses of genotype H1 evade recognition by vaccine-induced neutralizing antibodies targeting the linear haemagglutinin noose epitope. *Journal of General Virology* **90**, 2739-2745

37. Shi, J., Zheng, J., Huang, H., Hu, Y., Bian, J., Xu, D., and Li, F. (2011) Measles incidence rate and a phylogenetic study of contemporary genotype H1 measles

strains in China: is an improved measles vaccine needed? *Virus Genes* **43**, 319-326

38. TAMIN, A., ROTA, P., WANG, Z., HEATH, J., ANDERSON, L., and BELLINI, W. (1994) ANTIGENIC ANALYSIS OF CURRENT WILD-TYPE AND VACCINE STRAINS OF MEASLES-VIRUS. *Journal of Infectious Diseases* **170**, 795-801

39. Kuhne, M., Brown, D., and Jin, L. (2006) Genetic variability of measles virus in acute and persistent infections. *Infection Genetics and Evolution* **6**, 269-276

40. Santibanez, S., Niewiesk, S., Heider, A., Schneider-Schaulies, J., Berbers, G., Zimmermann, A., Halenius, A., Wolbert, A., Deitemeier, I., Tischer, A., and Hengel, H. (2005) Probing neutralizing-anti body responses against emerging measles viruses (MVs): immune selection of MV by H protein-specific antibodies? *Journal of General Virology* **86**, 365-374

41. Tahara, M., Ito, Y., Brindley, M., Ma, X., He, J., Xu, S., Fukuhara, H., Sakai, K., Komase, K., Rota, P., Plemper, R., Maenaka, K., and Takeda, M. (2013) Functional and Structural Characterization of Neutralizing Epitopes of Measles Virus Hemagglutinin Protein. *Journal of Virology* **87**, 666-675

42. Tahara, M., Ohno, S., Sakai, K., Ito, Y., Fukuhara, H., Komase, K., Brindley, M., Rota, P., Plemper, R., Maenaka, K., and Takeda, M. (2013) The Receptor-Binding Site of the Measles Virus Hemagglutinin Protein Itself Constitutes a Conserved Neutralizing Epitope. *Journal of Virology* **87**, 3583-3586

43. Pelegrin, M., Naranjo-Gomez, M., and Piechaczyk, M. (2015) Antiviral Monoclonal Antibodies: Can They Be More Than Simple Neutralizing Agents?

*Trends in Microbiology* **23**, 653-665

44. Buss, N., Henderson, S., McFarlane, M., Shenton, J., and de Haan, L. (2012) Monoclonal antibody therapeutics: history and future. *Current Opinion in Pharmacology* **12**, 615-622

45. Holliger, P., and Hudson, P. (2005) Engineered antibody fragments and the rise of single domains. *Nature Biotechnology* **23**, 1126-1136

46. Nelson, A. (2010) Antibody fragments Hope and hype. *Mabs* **2**, 77-83

47. YOKOTA, T., MILENIC, D., WHITLOW, M., and SCHLOM, J. (1992) RAPID TUMOR PENETRATION OF A SINGLE-CHAIN FV AND COMPARISON WITH OTHER IMMUNOGLOBULIN FORMS. *Cancer Research* **52**, 3402-3408

48. Griffiths, A., and Duncan, A. (1998) Strategies for selection of antibodies by phage display. *Current Opinion in Biotechnology* **9**, 102-108

49. HUSTON, J., MUDGETTHUNTER, M., TAI, M., MCCARTNEY, J., WARREN, F., HABER, E., and OPPERMANN, H. (1991) PROTEIN ENGINEERING OF SINGLE-CHAIN FV ANALOGS AND FUSION PROTEINS. *Methods in Enzymology* **203**, 46-+

50. ARGOS, P. (1990) AN INVESTIGATION OF OLIGOPEPTIDES LINKING DOMAINS IN PROTEIN TERTIARY STRUCTURES AND POSSIBLE CANDIDATES FOR GENERAL GENE FUSION. *Journal of Molecular Biology* **211**, 943-958

51. FRAME, K., and HU, W. (1990) THE LOSS OF ANTIBODY PRODUCTIVITY IN CONTINUOUS CULTURE OF HYBRIDOMA CELLS. *Biotechnology and Bioengineering* **35**, 469-476

52. Huhalov, A., and Chester, K. (2004) Engineered single chain antibody fragments for radioimmunotherapy. *Quarterly Journal of Nuclear Medicine and Molecular Imaging* **48**, 279-288

53. OSHANNESSY, D., BRIGHAMBURKE, M., SONESON, K., HENSLEY, P., and BROOKS, I. (1993) DETERMINATION OF RATE AND EQUILIBRIUM BINDING CONSTANTS FOR MACROMOLECULAR INTERACTIONS USING SURFACE-PLASMON RESONANCE - USE OF NONLINEAR LEAST-SQUARES ANALYSIS-METHODS. *Analytical Biochemistry* **212**, 457-468

54. HORTON, R., CAI, Z., HO, S., and PEASE, L. (1990) GENE-SPLICING BY OVERLAP EXTENSION - TAILOR-MADE GENES USING THE POLYMERASE CHAIN-REACTION. *Biotechniques* **8**, 528-&

55. Ahmad, Z., Yeap, S., Ali, A., Ho, W., Alitheen, N., and Hamid, M. (2012) scFv Antibody: Principles and Clinical Application. *Clinical & Developmental Immunology*

56. Federoff, H. (2009) Development of Vaccination Approaches for the Treatment of Neurological Diseases. *Journal of Comparative Neurology* **515**, 4-14

57. Campana, V., Zentilin, L., Mirabile, I., Kranjc, A., Casanova, P., Giacca, M., Prusiner, S., Legname, G., and Zurzolo, C. (2009) Development of antibody fragments for immunotherapy of prion diseases. *Biochemical Journal* **418**, 507-515

58. Donofrio, G., Heppner, F., Polymenidou, M., Musahl, C., and Aguzzi, A. (2005) Paracrine inhibition of prion propagation by anti-PrP single-chain Fv miniantibodies. *Journal of Virology* **79**, 8330-8338

59. Kim, Y., Neelamegam, R., Heo, M., Edwardraja, S., Paik, H., and Lee, S. (2008) Improving the productivity of single-chain Fv antibody against c-Met by rearranging the order of its variable domains. *Journal of Microbiology and Biotechnology* **18**, 1186-1190

60. Buhler, P., Wetterauer, D., Gierschner, D., Wetterauer, U., Beile, U., and Wolf, P. (2010) Influence of Structural Variations on Biological Activity of Anti-PSMA scFv and Immunotoxins Targeting Prostate Cancer. *Anticancer Research* **30**, 3373-3379

61. Zhou, Y., Goenaga, A., Harms, B., Zou, H., Lou, J., Conrad, F., Adams, G., Schoeberl, B., Nielsen, U., and Marks, J. (2012) Impact of Intrinsic Affinity on Functional Binding and Biological Activity of EGFR Antibodies. *Molecular Cancer Therapeutics* **11**, 1467-1476

62. BIRD, R., and WALKER, B. (1991) SINGLE CHAIN ANTIBODY VARIABLE REGIONS. *Trends in Biotechnology* **9**, 132-137

63. LAKE, D., LAM, K., PENG, L., and HERSH, E. (1994) MOLECULAR-CLONING, EXPRESSION AND MUTAGENESIS OF AN ANTIINSULIN SINGLE-CHAIN FV (SCFV). *Molecular Immunology* **31**, 845-856

64. SHARON, J. (1990) STRUCTURAL CORRELATES OF HIGH ANTIBODY-AFFINITY - 3 ENGINEERED AMINO-ACID SUBSTITUTIONS CAN INCREASE THE AFFINITY OF AN ANTI-PARA-AZOPHENYLARSONATE ANTIBODY 200-FOLD. *Proceedings of the National Academy of Sciences of the United States of America* **87**, 4814-4817

65. Kjaer, S., Wind, T., Ravn, P., Ostergaard, M., Clark, B., and Nissim, A. (2001) Generation and epitope mapping of high-affinity scFv to eukaryotic elongation factor 1A by dual application of phage display. *European Journal of Biochemistry* **268**, 3407-3415

# Acknowledgements

First I would like to express my deepest gratitude to my supervisor Prof. Katsumi Maenaka for accepting me as a graduate student in his laboratory. I am indebted to him for his kind consideration to complete my PhD thesis in time. His patience, motivation and overall supportive guidance always inspired me to continue my study. I am also very much grateful to him for his valuable discussions and suggestions in every step to take challenges as well as to build up my future career.

I want to express my sincere gratitude to Associate Prof. Toyoyuki Ose, Assistant Profs. Kimiko Kuroki and Atsushi Furukawa for their kind support during my study period.

I would like to thank my mentor Dr. Takashi Tadokoro for the guidance of this study.

My sincere thanks to Prof. Shin-ichi Nakagawa and Lecturer Dr. Hiroshi Maita for his kind contribution to review my thesis and evaluate my research.

I would like to give thanks to Dr. Takao Hashiguchi and Prof. Yusuke Yanagi, Department of Virology, Faculty of Medicine, Kyushu University and also my thanks to Drs. Maino Tahara and Makoto Takeda, Department of Virology III, The National Institute of Infectious Diseases (NIID) for providing 2F4 monoclonal antibody and useful discussions.

My sincere thanks to Dr. Hideo Fukuhara for giving me the plasmid to start my research.

I would like to give thanks to Atsutoshi Imai, Koki Yoshida, Natsumi Sugimura and Mizuki Saito for helping to prepare materials and the experiments. My deepest thanks to Ami Takahashi for her kind support and try to understand me, which always encourage me to concentrate in my research.

I am also expressing my gratefulness to Sumiko Saito and Megumi Fuji for their kind

welcome response. Very special thanks to Sumiko Saito for her kind assistance and help to lead my life smoothly. I am personally grateful to her, for her kind supportive contribution with me.

I want to give thanks to all teachers, staffs and students in Maenaka Lab for their kindness.

I am grateful to my family to support me during my study period.

Finally, my deepest gratefulness goes to my beloved mother for her continuous love, support and unselfish blessing throughout my life.

Above all I want to give thanks the almighty Allah, for accepting my prayers for giving me the strength to go ahead.