# CHARACTERIZATION OF RECOMBINANT ANTIBODIES TARGETING HIV-1 CAPSID PROTEIN (P24): TOWARDS THE DEVELOPMENT OF ANTIBODY-BASED THERAPY AGAINST HIV/AIDS

**SITI AISYAH BINTI MUALIF**

**UNIVERSITI SAINS MALAYSIA**

**2017**

# CHARACTERIZATION OF RECOMBINANT ANTIBODIES TARGETING HIV-1 CAPSID PROTEIN (P24): TOWARDS THE DEVELOPMENT OF ANTIBODY-BASED THERAPY AGAINST HIV/AIDS

**by**

**SITI AISYAH BINTI MUALIF**

**Thesis submitted in the fulfillment of the requirement for the degree of Doctor of Philosophy**

**April 2017**

# ACKNOWLEDGEMENT

The completion of this thesis would not have been possible without the support of a lot of people. In particular I would like to thank:

Dr. Syed Atif Ali for accepting me as a PhD student and gave me a great chance of learning and growing as a researcher. Prof. Dr. Narazah for being supportive and willing to listen and discuss. My colleagues with whom I spent great time: Mohd Tasyriq, Chew Yik Wei, Mohd Alif, and Ronald Teow. Thank you for creating a nice ambience in the lab and being helpful whenever I needed assistance. All the interns and trainees that helped me with the work, you know who you are. I wish you all the best for your future! All the laboratory staff: Ms. Nurdianah Harif Fadzilah, Ms. Ira Maya Sophia Nordin, Mrs. Halianis Yusoff, Ms. Nurandlia Mohamad Koldaie, Mr. Ahmad Farid Asmail@Ismail, and Mrs. Rafedah Abas. Thank you so much for the continuous helping hands.

FRGS grant (203/CIPPT/67 11206) from the Ministry of Higher Education of Malaysia and Research Student Fund (USM/IPPT/2000/G-2/xiv) from AMDI, USM for financial supports for the research project. Not forgetting the MOHE scholarship (KPT (BS) 841003015520) for me to complete the study all these years.

My parents, whom always held my hands till end. You always believed in me and gave me the opportunity to make you happy and proud with endless love, trust, faith and support. My lovely husband and baby, for sticking with me until the final lane. I hope the sacrifices and efforts would be paid back with this.

## TABLE OF CONTENT

**ACKNOWLEDGEMENT** ii
**TABLE OF CONTENTS** iii
**LIST OF TABLES** ix
**LIST OF FIGURES** x
**LIST OF ABBREVIATIONS** xiv
**ABSTRAK** xvii
**ABSTRACT** xix

**CHAPTER 1: INTRODUCTION**
1.1 Background of the study 1
1.1.1 HIV and AIDS 1
1.1.2 HIV Pathogenesis 2
1.1.3 Developing vaccines and new HIV therapies 7
1.1.4 Antibody molecule 14
1.1.5 Generation of scFv antibodies 17
1.1.6 Phage display technology 20
1.1.7 Applications of phage display library 29
1.1.8 Expression systems for the production of scFv antibodies 31
1.2 Problem statement 35
1.3 Objectives of the study 36
1.4 Significance of the study 37

**CHAPTER 2: MATERIALS AND METHODS**
2.1 Materials 38
2.2 Methods: Experimental strategy 39
2.3 Cloning, expression and purification of HIV-1 p24 protein 41
2.3.1 Preparation of chemically competent cells 42
2.3.2 Transformation efficiency 43
2.3.3 Cloning of *p24* gene into pSA-6His-RIL vector 44
2.3.3(a) Construction of pSA-Hp24-6His-RIL vector 44

2.3.3(b) Restriction endonuclease of pSA-6His-RIL vector and *p24* gene 46
2.3.3(c) DNA extraction from agarose gels 46
2.3.3(d) Ligation of *p24* into pSA-6His-RIL 47
2.3.3(e) Transformation of pSA-Hp24-6His-RIL into *E. coli* DH5α competent cells 49
2.3.3(f) Colony PCR 49
2.3.3(g) Plasmid DNA extraction 51
2.3.3(h) Restriction enzyme analysis and DNA sequencing 52
2.3.4 Expression of recombinant HIV-1 p24 protein 53
2.3.4(a) SDS-PAGE 53
2.3.4(b) Preparation of chemically competent cells 53
2.3.4(c) Preparation of NiCo21-pACYCRIL chemically competent cells 53
2.3.4(d) Transformation of NiCo21(DE3) competent cells with pSA-Hp24-6His-RIL clone 54
2.3.4(e) Protein extraction 54
2.3.4(f) SDS-PAGE 56
2.3.4(g) Western blot analysis 56
2.3.5 Purification of recombinant HIV-1 p24 protein: Spin column method 58
2.3.6 Purification of recombinant HIV-1 p24 protein: FPLC method 58
2.3.7 Dialysis and protein concentration 59

2.4 Construction of an antibody phage display library against HIV-1 p24 protein 61
2.4.1 Total RNA isolation from hybridoma cells (ATCC, #31-90-25) 63
2.4.2 Purification of total RNA with Lithium chloride 64
2.4.3 Generation of variable antibody fragments 64
2.4.3(a) First strand cDNA synthesis from total RNA 64
2.4.3(b) Construction of scFv repertoire 68
2.4.3(b)(i) First round of PCR 68
2.4.3(b)(ii) Second round of PCR 72

2.4.3(b)(iii) Overlap extension PCR 73
2.4.4 pHEN2 phagemid modifications 75
2.4.4(a) Cloning of RIL array into pHEN2 phagemid 75
2.4.4(b) Cloning of *sacB* and *ccdB* genes into pHEN2-RIL 77
2.4.5 Preparation of M13K07 helper phage stock 79
2.4.6 Preparation of chemically competent TG1 cells 80
2.4.7 Cloning of the scFv genes into pHEN2 phagemid 81
2.4.7(a) Restriction/digestion of the scFv and phagemid pHEN2 81
2.4.7(b) Ethanol precipitation 83
2.4.7(c) Library ligation and transformation 83
2.4.8 Affinity selection of the phage display library against recombinant HIV-1 p24 antigen 87
2.4.9 Analysis of phage display library by ELISA 90
2.4.9(a) Identification of polyclonal phage 90
2.4.9(b) Identification of monoclonal phage 93
2.4.10 Individual phage clone preparation 94
2.4.11 Expression and purification of soluble antibody fragments 94

2.5 Expression of anti-p24 scFv antibodies in bacteria and mammalian expression systems 95
2.5.1 Cloning of anti-p24 scFv into pSA-6His vector 97
2.5.1(a) Construction of pSA-MBPTEV-scFv46 vector 97
2.5.1(a)(i) PCR amplification of pSA-6His vector and MBP-TEV 97
2.5.1(a)(ii) Cloning of MBP-TEV-scFv46 into pSA-6His vector 100
2.5.1(b) Cloning of *sacB* gene into pSA-MBP-TEV-6His vector 105
2.5.1(c) Construction of pSA-MBPTEV-scFv-6His clones 106
2.5.2 Cloning of anti-p24 scFv into pcDNA3.3 vector 109
2.5.2(a) Cloning of scFv46 into pcDNA3.3 vector 109
2.5.2(b) Cloning of *sacB* gene into pcDNA3.3 vector 111

2.5.2(c) Construction of pcDNA3.3-scFv clones 113
2.5.3 Cloning of anti-p24 scFv into pEF1α vector 116
2.5.4 Expression of anti-p24 scFv in bacteria system 119
2.5.4(a) Purification of anti-p24 scFv fusion protein using immobilized metal affinity chromatography (IMAC): Spin column method 1200
2.5.4(b) Protein concentration and protein lyophilization 120
2.5.4(c) Polymerization inhibition assay 121
2.5.5 Expression of anti-p24 scFv in mammalian cell system 122
2.5.5(a) Generation of stable cell line 122
2.5.5(b) Transient expression 122
2.5.5(b)(i) Calcium phosphate method 122
2.5.5(b)(ii) X-tremeGene transfection reagent method 123
2.5.5(b)(iii) Neon® Transfection system – Electroporation method 124
2.5.5(c) Preparation of HIV-1 stock 125
2.5.5(d) HIV-1 infection of the transfected cells 126
2.5.5(e) MAGI infectivity assay – HIV-1 inhibition assay 126
2.5.5(f) Triton X-100 lysis method 127
2.5.5(g) Maintaining the Jurkat T cells (ATCC® TIB-152) 127
2.5.5(h) Maintaining the 293T cells (ATCC® CRL-3216™) 128

**CHAPTER 3: RESULTS**
3.1 Cloning, expression and purification of HIV-1 p24 protein 129
3.1.1 Construction of pSA-Hp24-6His-RIL clone 129
3.1.2 Expression of p24 from pSA-Hp24-6His vectors with or without rare tRNA genes 131
3.1.3 Purification of recombinant HIV-1 p24 protein 136

3.2 Construction of an antibody phage display library against HIV-1 p24 protein 142
3.2.1 Cloning of RIL array into pHEN2 phagemid vector 142

3.2.2 Cloning of sacB and ccdB genes into pHEN2-RIL phagemid vector 146
3.2.3 Helper phage stock preparation (M13KO7) 154
3.2.4 Chemically competent TG1 cells 154
3.2.5 Isolation of total RNA from p24 hybridoma (ATCC, 31-90-25) 154
3.2.6 Construction of scFv repertoire 156
3.2.7 Cloning of scFv into pHEN2-RIL 160
3.2.8 Affinity selection of phage display library 164
3.2.9 Identification of polyclonal and monoclonal phage specific towards recombinant HIV-1 p24 168
3.2.10 Analysis of clonal integrity of anti-p24 scFv clones 172
3.2.11 Anti-p24 scFv expression 177

3.3 Expression of anti-p24 scFv proteins 179
3.3.1 Cloning of anti-p24 scFv into pSA-MBP-6His vector 179
3.3.1(a) Construction of pSA-MBPTEV-scFv46-6His clone 179
3.3.1(b) Construction of pSA-MBPTEV-sacB-6His clone 189
3.3.1(c) Construction of pSA-MBPTEV-scFv-6His clone 195
3.3.2 Cloning of anti-p24 scFv into pcDNA3.3-6His vector 202
3.3.2(a) Construction of pcDNA3.3-scFv46-6His clone 202
3.3.2(b) Construction of pcDNA3.3-sacB-6His clone 207
3.3.2(c) Construction of pcDNA3.3-scFv-6His clones 210
3.3.3 Expression of anti-p24 scFvs in pSA-MBPTEV-6His expression vector 213
3.3.3(a) Expression of anti-p24 scFvs in Shuffle T7 Express strain 213
3.3.3(b) Purification of anti-p24 scFv protein using spin column method of cobalt resin and FPLC 216
3.3.3(c) HIV-1 p24 polymerization kinetics assay 220
3.3.3(d) HIV-1 p24 direct ELISA 223
3.3.4 Expression of anti-p24 scFvs in pcDNA3.3-6His expression vector 225
3.3.4(a) Expression of anti-p24 scFv46 in 293T cells 227

3.3.4(b) Expression of anti-p24 scFvs in Jurkat T cells 227
3.3.4(b)(i) Generation of stable cell line 227
3.3.4(c) MAGI infectivity assay 230
3.3.5 Expression of anti-p24 scFv in pEF1α expression vector 232
3.3.5(a) Expression of anti-p24 scFv in 293T cells under EF1α promoter 232
3.3.5(b) Expression of anti-p24 scFv16 in Jurkat T cells 236
3.3.5(c) MAGI infectivity assay 239

**CHAPTER 4: DISCUSSIONS** 241
**CHAPTER 5: CONCLUSIONS** 257
**CHAPTER 6: RECOMMENDATIONS** 258
**CHAPTER 7: LIST OF REFERENCES** 259
**CHAPTER 8: APPENDICES** 270

## LIST OF TABLES

| | | Page |
|---|---|---|
| Table 2.3.1. | Oligonucleotides used to construct and verify pSA-Hp24-6His and pSA-Hp24 6His-RIL expression vectors | 50 |
| Table 2.4.1. | First-strand cDNA synthesis mixture - #1 | 66 |
| Table 2.4.2. | First-strand cDNA synthesis mixture - #2 | 66 |
| Table 2.4.3. | Oligonucleotides used to amplify and construct scFv | 69 |
| Table 2.4.4. | Oligonucleotides used to construct and verify pHEN2-RIL and pHEN2-scFv vectors | 78 |
| Table 2.4.5. | Restriction/digestion of scFv and pHEN2 | 82 |
| Table 2.5.1. | Oligonucleotides used to construct and verify pSA-MBPTEV-scFv-6His and pcDNA3.3-scFv-6His expression vectors | 98 |
| Table 2.5.2. | PCR components of pSA-6His vector and MBP-TEV | 99 |
| Table 2.5.3 | Restriction/digestion of the vector and insert | 102 |
| Table 2.5.4. | Ligation reactions of the scFv inserts and pSA-MBPTEV-6His vector | 107 |
| Table 2.5.5. | PCR components of pcDNA3.3 vector and scFv46 | 110 |
| Table 2.5.6. | Ligation reactions of the scFv inserts and pcDNA3.3 vector | 115 |
| Table 2.5.7. | Oligonucleotides used to construct and verify pEF1α-GFP-2A-scFv6His expression vectors | 118 |
| Table 3.1.1. | Yield and percentage purity comparison of recombinant HIV-1 p24 from NiCo21 transformed with either pSA-Hp24-6His + pACYC-RIL or pSA-Hp24-6His-RIL vectors | 141 |
| Table 3.2.1. | Small scale ligation results | 163 |
| Table 3.2.2. | Library size determination | 163 |
| Table 3.3.1. | Recombinant MBP-scFvs protein yields | 219 |
| Table 3.3.2. | Assembly rate and inhibition of HIV-1 p24 polymerization | 222 |

# LIST OF FIGURES


| | | Page |
|---|---|---|
| Figure 1.1.1. | HIV-1 genome | 3 |
| Figure 1.1.2. | HIV replication cycle | 5 |
| Figure 1.1.3. | HIV life cycle and antiretroviral drugs | 9 |
| Figure 1.1.4. | Organization and structure of HIV-1 Gag and CA proteins | 11 |
| Figure 1.1.5. | Schematic representations of the antibody structure | 15 |
| Figure 1.1.6. | Recombinant antibody constructs | 18 |
| Figure 1.1.7. | Schematic presentation of phage display system | 21 |
| Figure 1.1.8. | Schematic presentation of biopanning | 23 |
| Figure 2.2.1. | Overview of experimental strategy in this study | 40 |
| Figure 2.3.1. | Construction of pSA-Hp24-6His-RIL vector | 45 |
| Figure 2.3.2. | Schematic representation of pSA-Hp24-6His-RIL clone | 48 |
| Figure 2.4.1. | Schematic overview of the anti-p24 scFv antibody phage display library construction | 62 |
| Figure 2.4.2. | Construction of pHEN2-RIL vector | 76 |
| Figure 2.4.3. | Schematic representation of pHEN2-scFv-RIL clone | 86 |
| Figure 2.5.1. | Schematic overview of the anti-p24 scFv antibody expression | 96 |
| Figure 2.5.2. | Schematic representation of pSA-MBP-TEV-6His and pSA-MBP-TEV-sacB-6His vectors | 104 |
| Figure 2.5.3. | Schematic representation of pcDNA3.3-sacB-6His vector. | 112 |
| Figure 2.5.4. | Schematic representation of pEF1α-GFP-2A-scFv clone | 117 |
| Figure 3.1.1. | Restriction enzyme digestion of plasmids and verification of pSA-Hp24-6His-RIL clone | 130 |
| Figure 3.1.2. | Expression of p24 from pSA-Hp24-6His vectors with or without rare tRNA genes array | 132 |
| Figure 3.1.3. | Densitometry analysis of recombinant HIV-1 P24 protein expression | 133 |
| Figure 3.1.4. | Effect of rare tRNA supplementation on P24 expression in NiCo21(DE3) *E. coli* | 135 |
| Figure 3.1.5. | Growth curves of the cells harboring recombinant HIV-1 p24 protein | 137 |

| | | |
|---|---|---|
| Figure 3.1.6. | Production and purification of HIV-1 P24 protein | 139 |
| Figure 3.2.1. | Construction of pHEN2-RIL plasmid | 143 |
| Figure 3.2.2. | Verification of pHEN2-RIL clone | 145 |
| Figure 3.2.3 | Preparation of *sacB* gene and pHEN2-RIL vector | 147 |
| Figure 3.2.4. | Clones verification for pHEN2-sacB RIL | 149 |
| Figure 3.2.5. | Restriction digestion of pHEN2-RIL vector and *ccdB* gene | 151 |
| Figure 3.2.6. | Verification of pHEN2-ccdB RIL clones | 153 |
| Figure 3.2.7. | Isolation of total RNA | 155 |
| Figure 3.2.8. | Optimization of first round PCR | 157 |
| Figure 3.2.9. | Construction of scFv repertoire | 159 |
| Figure 3.2.10. | Restriction of pHEN2 phagemid and scFv | 161 |
| Figure 3.2.11. | Analysis of clonal integrity by PCR amplification | 165 |
| Figure 3.2.12. | Analysis of clonal diversity by *BstN*I fingerprinting | 167 |
| Figure 3.2.13. | Identification of polyclonal phage by ELISA | 170 |
| Figure 3.2.14. | Identification of monoclonal phage by ELISA | 171 |
| Figure 3.2.15. | Plasmid DNA extraction and PCR of pHEN2-scFv RIL clones | 173 |
| Figure 3.2.16. | Analysis of clonal integrity of anti-p24 scFv clones | 174 |
| Figure 3.2.17. | Sequence alignment of anti-p24 scFv clones | 176 |
| Figure 3.2.18. | Expression of anti-p24 scFv in T7 Shuffle Express strain | 178 |
| Figure 3.3.1. | Amplification of pSA-6His vector, MBP-TEV, and scFv46 insert | 180 |
| Figure 3.3.2. | Restriction of pSA-6His vector, MBP-TEV, and scFv46 inserts | 182 |
| Figure 3.3.3. | Production of MBPTEV-scFv46 | 184 |
| Figure 3.3.4. | Colony PCR for verification of pSA-MBPTEV-scFv46 clones | 186 |
| Figure 3.3.5. | Restriction enzyme analysis of pSA-MBP-scFv46 | 188 |
| Figure 3.3.6. | Restriction of pSA-MBPTEV-6His, pcDNA3.3-6His vectors, and *sacB* insert | 190 |
| Figure 3.3.7. | Colony PCR for verification of pSA-MBPTEV-sacB clones | 192 |

| | | |
|---|---|---|
| Figure 3.3.8. | Restriction enzyme digestion of pSA-MBPTEV-sacB plasmids for clone verification. | 194 |
| Figure 3.3.9. | Restriction enzyme digestion of pSA-MBPTEV-6His, pcDNA3.3-6His vectors, and scFv inserts | 196 |
| Figure 3.3.10. | Colony PCR for verification of pSA-MBP-scFv clones | 198 |
| Figure 3.3.11. | Restriction enzyme digestion of pSA-MBP-scFv plasmids for clone verification | 201 |
| Figure 3.3.12. | Amplification of pcDNA3.3 vector and scFv46 insert | 203 |
| Figure 3.3.13. | Restriction enzyme digestion of pcDNA3.3 vector and scFv46 insert | 204 |
| Figure 3.3.14. | Restriction analysis of pcDNA3.3-scFv46. | 206 |
| Figure 3.3.15. | Colony PCR for verification of pcDNA3.3-sacB clones | 208 |
| Figure 3.3.16. | Restriction analysis of pcDNA3.3-sacB plasmids for clone verification | 209 |
| Figure 3.3.17. | Colony PCR for verification of pcDNA3.3-scFvs clones | 211 |
| Figure 3.3.18. | Restriction analysis of pcDNA3.3-scFv plasmids for clone verification | 212 |
| Figure 3.3.19. | Expression of anti-p24 scFvs in Shuffle T7 Express strain | 214 |
| Figure 3.3.20. | Expression of anti-p24 scFv in Shuffle T7 Express strain, IPTG optimization | 215 |
| Figure 3.3.21. | Recombinant MBP-scFv yields and purification | 218 |
| Figure 3.3.22. | Inhibition of HIV-1 P24 polymerization in vitro | 221 |
| Figure 3.3.23. | MBP-anti p24 scFv binding with recombinant and HIV-derived -p24 | 224 |
| Figure 3.3.24. | Expression of anti-p24 scFv46 in 293T cells | 226 |
| Figure 3.3.25. | Expression of p24 scFvs in stably transfected Jurkat T cells and genomic DNA extraction | 228 |
| Figure 3.3.26. | HIV-1 infection inhibition in Jurkat T cells | 231 |
| Figure 3.3.27. | Transfection of 293T cells with pEF1α-GFP2A-scFv16, -46, and GFP-2A-VSV-G plasmids | 233 |
| Figure 3.3.28. | Western blot analysis of 293T cells transfected with pEF1α-GFP2A-scFv16, -46, and GFP-2A-VSV-G plasmids | 234 |

| | | |
|---|---|---|
| Figure 3.3.29. | Western blot analysis of 293T cells transfected with pEF1α-GFP2A-scFv16, -46, and GFP-2A-VSV-G plasmids; the cell pellets | 235 |
| Figure 3.3.30. | Transient transfection of Jurkat T cells with plasmids containing anti-p24 scFvs | 238 |
| Figure 3.3.31. | Inhibition of HIV-1 infection by anti-p24 scFvs | 240 |

## LIST OF ABBREVIATIONS

| | |
|---|---|
| **2X** | Two times |
| **4X** | Four times |
| **A600** | Abosrbance at 600 nm |
| **Ab** | Antibody |
| **Amp** | Ampicillin |
| **CA** | Capsid |
| **Cam** | Chloramphenicol |
| **CDR** | complementarity determining region |
| **CMV** | Cytomegalovirus |
| **DMSO** | dimethyl sulfoxide |
| **DNA** | Deoxy Ribonucleic Acid |
| **DNase** | deoxyribonuclease |
| **dNTP** | deoxynucleoside triphosphate |
| **$ddH_2O$** | Double-distilled water |
| **ELISA** | enzyme-linked immunosorbent assay |
| **Fab** | Antigen binding fragment |
| **$H_2O$** | Water |
| **HCl** | Hydrochloric acid |
| **HIV** | Human Immunodefeciency Virus |
| **HSV** | Herpes simplex virus |
| **Ig** | Immunoglobulin |
| **IN** | Integrase |
| **IPTG** | Isopropyl-beta-D-thiogalactopyranoside |
| **Kan** | Kanamycin |
| **kb** | kilo base |
| **kDa** | kiloDalton |
| **L** | liter |
| **LB** | Luria Bertani medium |
| **MA** | Matrix |
| **mAb** | Monoclonal antibody |
| **min** | minute/s |
| **mL** | milli liter |

| | |
|---|---|
| **mRNA** | messenger RNA |
| **MW** | molecular weight |
| **NC** | Nuclecapsid |
| **NEB** | New England Biolabs |
| **nm** | nanometer |
| **nM** | nanomolar |
| **ORF** | Open reading frame |
| **PBS** | Phosphate Buffered Saline |
| **PCR** | Polymerase chain reaction |
| **PEG** | Polyethylene glycol |
| **pH** | Potential hydrogeni |
| **pmol** | pico mole |
| **PMSF** | phenyl methyl sulfonyl fluoride |
| **PR** | Protease |
| **RNA** | ribonucleic acid |
| **RSV** | Respiratory syncytial virus |
| **RT** | room temperature |
| **s** | second/s |
| **SB** | Super Broth medium |
| **scFv** | single-chain variable fragment |
| **SOB** | Super Optimal Broth |
| **SOC** | Super optimal broth with Catabolic repressor |
| **Strep** | Streptomycin |
| **TAE** | Tris/acetate/EDTA (buffer) |
| **Taq** | Thermus aquaticus |
| **Tet** | Teracycline |
| **TB** | Terrific Broth medium |
| **Tm** | Melting temperature |
| **Tris** | Tris(hydroxymethyl)aminomethane |
| **tRNA** | transfer RNA |
| **UV** | Ultraviolet |
| **V** | volt |
| **$V_H$** | Variable heavy chain |

| | |
|---|---|
| **$V_L$** | Variable light chain |
| **v/v** | volume/volume |
| **VZV** | Varicella zoster virus |
| **w/v** | weight/volume |
| **X-Gal** | 5-bromo-4-chloro-3indolyl-β-D-galactopyranoside |
| **YT** | 2xYT medium |
| **YTAG** | 2xYT with ampicillin and 1% glucose |
| **B-ME** | Beta-mercaptoethanol |
| **µL** | micro liter |
| **µm** | micrometer |
| **µM** | micromolar |

# PENCIRIAN ANTIBODI REKOMBINAN YANG MENSASARKAN PROTEIN KAPSID HIV-1 (P24): KE ARAH PEMBANGUNAN ANTIBODI TERAPEUTIK TERHADAP HIV/AIDS

## ABSTRAK

Virus imunodifisiensi manusia (HIV) adalah agen penyebab sindrom kurang daya tahan terhadap penyakit (AIDS). Peningkatan morbiditi dan kematian akibat HIV/AIDS dalam beberapa dekad kebelakangan ini telah mencetuskan perhatian untuk memerangi penyakit berkenaan. Walau bagaimanapun, penyelidikan yang dijalankan secara berterusan didapati masih gagal untuk membasmi jangkitan HIV/AIDS. Pengenalan terhadap terapi *anti-retroviral* yang sangat aktif (HAART) pada awal tahun 1990 telah mengurangkan kadar kematian HIV/AIDS, namun ia mewujudkan strain HIV yang mempunyai rintangan terhadap dadah/ ubat-ubatan. Oleh itu, terdapat keperluan yang mendesak untuk membangunkan kaedah terapeutik baharu yang lebih baik. *HIV-1 kapsid protein* (p24) memainkan peranan penting dalam kedua-dua peringkat replikasi awal dan lewat virus HIV-1. Molekul kecil *Inhibitor* dan peptida yang mensasarkan p24 membuktikan jangkitan virus berkenaan dapat dihalang. Walau bagaimanapun, faktor perembesan pantas dan ketoksikan molekul/peptide tersebut merupakan kelemahan utama yang berkaitan dengan kaedah terapeutik di atas. Potensi antibodi monoklon (mAbs) yang berupaya mensasarkan p24 telah diterokai. Antibodi monoklon berkenaan dapat dibangunkan sebagai modaliti terapeutik yang baharu. Walau bagaimanapun, antibodi monoklon semula jadi atau yang diperoleh dari *hybridoma* merupakan molekul bersaiz besar yang sukar untuk menembusi sel. Teknologi DNA rekombinan membolehkan kejuruteraan antibodi dibangunkan dalam pelbagai format. Oleh itu, matlamat kajian ini adalah untuk menjana, mencirikan, dan

menilai antibodi rekombinan yang mensasarkan HIV-1 p24. Antibodi rekombinan anti-p24 dijana dari kombinasi pelbagai kumpulan domain yang diklon daripada sel *hybridoma* dan seterusnya dihasilkan pada permukaan filamen bakteriofaj. *ScFvs* rekombinan yang bertindak secara spesifik terhadap p24 HIV-1 telah diperoleh dan dikenal pasti. Daripada 50 klon, tiga scFvs yang spesifik telah dikenal pasti melalui ujian ELISA. *scFvs* tersebut telah disahkan melalui *competitive* ELISA dan klon-klon berkenaan kemudiannya dihasilkan di dalam *E. coli*. *ScFvs* rekombinan didapati berupaya menghalang pempolimeran p24 secara *in vitro* dan replikasi HIV di dalam beberapa jujukan Jurkat T sel apabila dihasilkan sebagai antibodi intrasel *(intrabodies*). *ScFvs* anti-p24 yang dihasilkan daripada kajian ini mempunyai potensi untuk dibangunkan sebagai kaedah terapeutik baharu yang berasaskan antibodi terhadap HIV.

# CHARACTERIZATION OF RECOMBINANT ANTIBODIES TARGETING HIV-1 CAPSID PROTEIN (P24): TOWARDS THE DEVELOPMENT OF ANTIBODY-BASED THERAPY AGAINST HIV/AIDS

## ABSTRACT

Human immunodeficiency virus (HIV) is the causative agent of acquired immune deficiency syndrome (AIDS) disease. Increasing morbidity and mortality due to HIV/AIDS in decades has sparked an interest to combat HIV/AIDS. However, ongoing research against the HIV/AIDS pandemic has failed to completely eradicate the infection. Introduction of highly active anti-retroviral therapy (HAART) in early 1990s has reduced the death rate of HIV/AIDS, but it has also resulted in the development of drug-resistant strains of HIV. Therefore, there is a pressing need to develop new and improved therapeutic modalities. HIV-1 capsid protein (p24) plays important roles in both early and late stages of HIV-1 replication. Small molecule inhibitors and peptides targeting p24 have shown to inhibit viral infection. However, rapid clearance and toxicity are major drawbacks associated with the above-mentioned therapeutic modalities. The potential of monoclonal antibodies (mAbs) targeting p24 was discovered and found out that p24-targeting antibodies can be developed into novel therapeutic modalities. However, natural or hybridoma-derived mAbs are large molecules and difficult to engineer. Recombinant DNA technology allows the engineering of antibodies in multiple formats. Therefore, the aim of this study was to generate, characterize, and evaluate recombinant antibodies targeting HIV-1 p24. Recombinant anti-p24 antibodies were generated from a combinatorial library of variable domains cloned from a hybridoma cell line and subsequently expressed on the surface of filamentous bacteriophage. Recombinant scFvs reacting specifically with

HIV-1 p24 were isolated, expressed, and characterized. Out of 50 clones, three specific binders were identified via initial ELISA screening. Specificity of the binders was confirmed through competition studies and the selected clones were expressed in *E. coli*. The recombinant scFvs markedly inhibited p24 polymerization *in vitro* and HIV replication in Jurkat T cell lines when expressed as intracellular antibodies (intrabodies). The anti-p24 scFvs engineered in this study have potential to be developed into novel antibody–based therapeutics against HIV.

# CHAPTER 1: INTRODUCTION

## 1.1. Background of the study

### 1.1.1. HIV and AIDS

Human immunodeficiency virus (HIV) is categorized into two types: HIV-1 and HIV-2. The routes of transmission for both types are similar and are causative agents for acquired immunodeficiency disease syndrome (AIDS). HIV-1 is more pathogenic as compared to HIV-2 thus making it the more predominant virus. There are three HIV-1 groups which composed of M, N, and O groups. Among these groups, M is the main group that covers 90% of the HIV-1 infection. Recently, a new group was identified known as group P. Within group M, there are nine subtypes with 15-20% genetic variations (A, B, C, D, F, G, H, J, and K) in which Subtype C was identified as the cause of 50% of the total HIV-1 infections in 2004 (Kurth & Bannert, 2010).

HIV is transmitted by several ways; predominantly from sexual intercourse, from mother to child, intravenous drug injection or contaminated blood transfusion. In Malaysia, until 2015, the number of people living with HIV was 92,895 or almost 0.5% of the entire population (MOH reports). This number has increased from 91,362 cases of HIV infections at the end of 2010. Choy (2014) reported that the development of highly active antiretroviral therapy (HAART) in the 1990s had reduced or slowed down the death rate of the HIV/AIDS patients in Malaysia. The most-at-risk-populations for HIV transmission in Malaysia are, injecting drug users (IDUs), transgender people, sex workers, and migrant workers (Choy, 2014).

### 1.1.2. HIV Pathogenesis

The HIV-1 genome is approximately 9.8 kb in length that encode for structural, regulatory, and accessory proteins which flanked by repeated sequence known as long terminal repeats (LTRs) (**Figure 1.1.1**). By having these proteins, HIV-1 is considered a complex retrovirus. HIV-1 LTRs are composed of promoter and enhancer sequences as well as polyadenylation site. They are important for reverse transcription, integration and gene expression steps. The HIV genome contains nine genes. In addition to the *gag*, *pol*, and *env* genes coding for structural proteins (Matrix, Capsid, Nucleocapsid, p6) and enzymes (protease, reverse transcriptase, integrase), there are two regulatory (*tat* and *rev*) and four accessory genes (*vif*, *vpr*, *vpu*, and *nef*) which are present in the HIV as a complex retrovirus (Li et al., 2015).

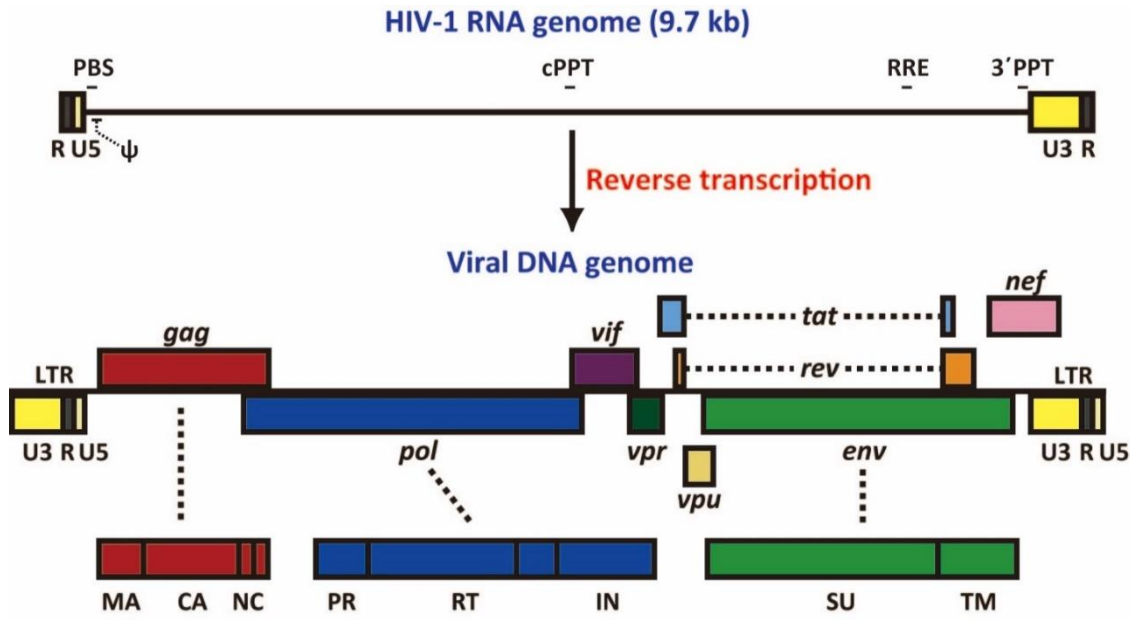


**Figure 1.1.1. HIV-1 genome.** HIV-1 genome consists of structural, regulatory and accessory genes flanked by the promoters in LTRs (Suzuki & Suzuki, 2011).

MA (matrix), CA (capsid), NC (nucleocapsid), PR (protease), RT(reverse transcriptase), IN (integrase), SU (gp120), TM (gp41).

Pathogenesis of the virus is attributed to the direct and indirect mechanism of all the viral genes. They are involved differently at each step of the viral replication – from the early stage until the release of the virus from the host cell (**Figure 1.1.2**). Firstly, the viral gp120 will recognize the host cell surface receptor, CD4. The glycoprotein undergoes conformational changes that allows binding to the co-receptor (either CCR5 or CXCR4, depending on the HIV tropism). This is followed by fusion with the cell membrane which is mediated by the transmembrane gp41 protein. The viral capsid is subsequently released into the cytoplasm. Once in the cell, the viral capsid is partially disassembled and reverse transcription takes place. This process is facilitated by the reverse transcriptase enzyme to form viral DNA. Viral DNA, p17 Gag, integrase, and Vpr are contained in the preintegration complex (PIC). The viral DNA is transported to the nucleus and with the assistance of integrase and Vpr protein, the DNA integrates with the host DNA to produce a provirus (Fauci, 2007).

In the nucleus, the provirus DNA is transcribed by the host RNA polymerase into RNA. This process is mediated by Tat that binds at the LTR sequence to promote transcription of longer copies of the viral genome. RNA splicing takes place either singly or multiply or otherwise remain unspliced. Singly spliced and multiply spliced RNA which are then exported from the nucleus are called virion proteins. This is assisted by the Rev protein. Meanwhile, the unspliced RNA is translated into viral RNA by the host ribosomes which are also released into the cytoplasm. During this step, the new viral RNA and proteins are brought together and move towards the plasma membrane. These components are together known as immature virion when released from the host cell. Finally, the capsid is formed

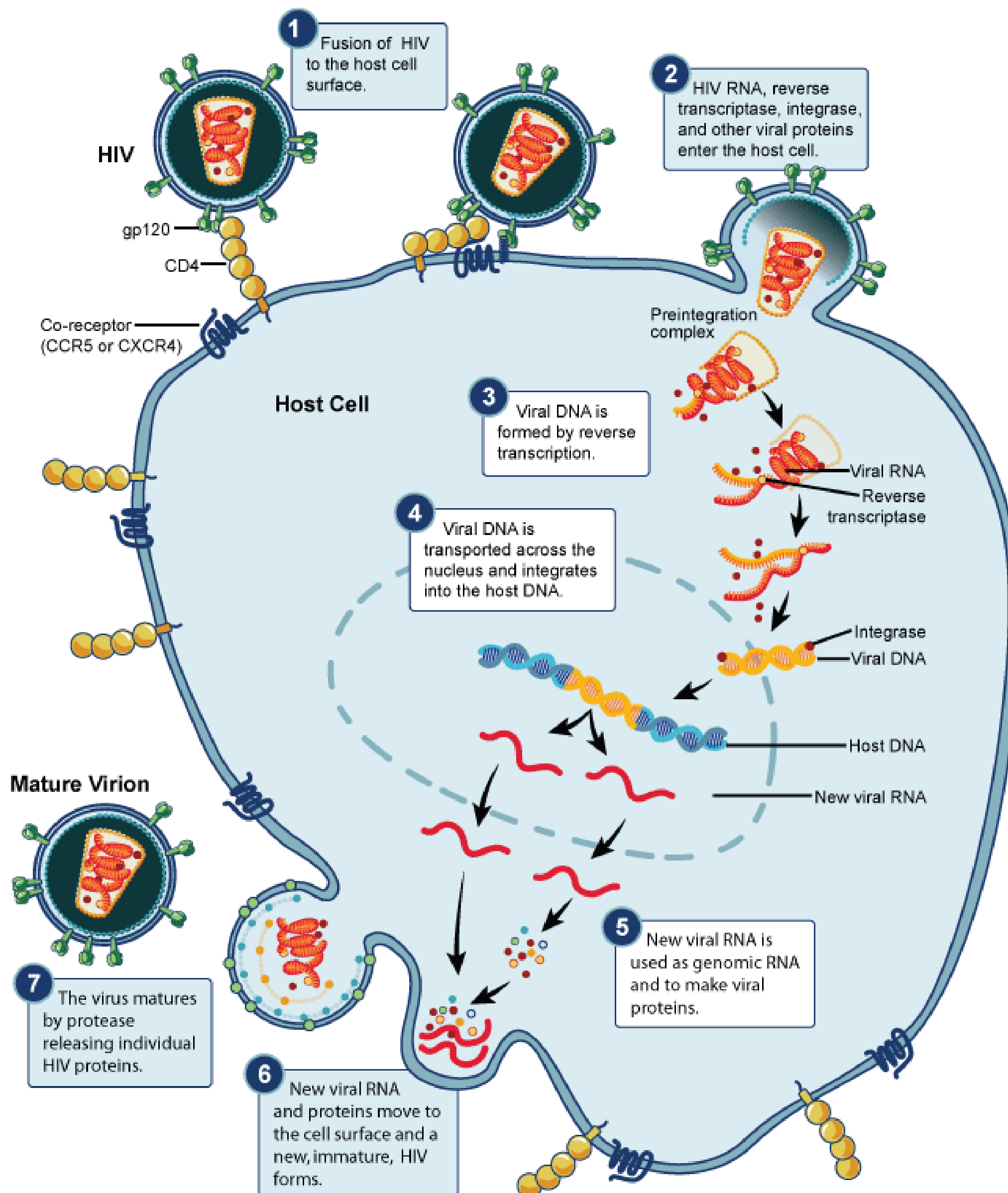


**Figure 1.1.2. HIV replication cycle.** HIV replication cycle consists of 6 essential steps: 1) host cell binding and entry, 2) uncoating of the capsid, 3) reverse transcription of the viral RNA, 4) integration of the viral DNA complex into host DNA, 5) virus protein synthesis and assembly, 6) exocytosis or storage of viral RNA in the host cell. The virus matures and starts infecting uninfected cells. Figure retrieved from http://www.niaid.nih.gov/SiteCollectionImages/topics/hivaids/hivReplicationCycle.gif.

by the P24 protein and the virus matures and becomes infectious to uninfected cells (Reitz & Gallo, 2010).

A marked increase in immune activation of the host is a feature of HIV infection, including both innate and adaptive immunity. These two immune systems are important components to eradicate the virus once the host is infected. Innate immune response also known as natural immune response triggers the typical general immune events against many pathogens with no specificity of the particular invader. Conversely, an adaptive immune response specifically attacks the invader. $CD4^+$ T cells are included in the adaptive immunity together with $CD8^+$ T cells and B cells. HIV is capable of manipulating the host immune system to its advantage. For instance, infected $CD4^+$ T cells will reach the lymph nodes, where activated T cells are located. Thus, the immune cells can be further infected resulting in depletion of $CD4^+$ T cells (Swanstrom & Coffin, 2012; Maartens et al., 2014).

The host cell has counteracting mechanisms to block the virus. Tetherin is a transmembrane protein of the host to inhibit the release of viral products. However, one of the viral proteins, Vpu, degrades tetherin to allow the release of viral particles. Another significant antiviral activity by the host is APOBEC-3G (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G). APOBEC is an intracellular host defense mechanism against retroviruses which inhibits replication of the viruses. However, the Vif protein of HIV prevents incorporation of APOBEC into the virions. Vif functions by depleting cytoplasmic APOBEC thus promotes degradation of APOBEC3G via proteasomal pathway. Researchers had discovered new intracellular antiviral mechanism mediated by the tripartite motif (TRIM) family. The alpha isoform of TRIM (TRIM5α) is a retrovirus restriction factor that provides an early block to retrovirus infection. It binds to viral capsid hexamers and inhibits capsid

uncoating and reverse transcription. The mechanism of TRIM5α antiviral activity is not yet reported in details (Poli & Erfle, 2010).

### 1.1.3. Development of vaccines and new HIV therapies

In the absence of antiretroviral therapy, HIV infection will lead to the development of AIDS. The therapy is not a cure for the infection but delays the symptoms from worsening. To eradicate the virus, a protective vaccine is required. The development of a HIV vaccine has been very challenging. The most advanced vaccine to date is known as RV144. It has been tested up to phase III by a Thai group. They have tested a combination of two vaccines, ALVAC® HIV as the primary vaccine and AIDSVAX® B/E as the booster, which were based on the virus strains commonly found in Thailand. Haynes et al. (2012), mentioned that the tested vaccine protected some volunteers. The mechanism involved was the binding of immunoglobulin G antibodies to variable 1 and 2 (V1/V2) regions of HIV-1 Env protein which resulted in non-functional Env protein.

Several HIV-1 enzymes have been targeted for drug development such as integrase, reverse transcriptase (RT), and protease (PR) enzymes. Antiretroviral drugs have been developed since the introduction of zidovudine (AZT) at the National Cancer Institute in 1987. AZT is one of the nucleoside reverse transcriptase inhibitors (NRTIs) drugs besides retrovir. This group of drug inhibits reverse transcriptase activity during viral DNA production which acts as monotherapy to the patients. Another class of antiretroviral drugs is non-nucleoside reverse transcription inhibitors (NNRTIs) that also block the reverse transcription. The approved drugs in this class include nevirapine, delavirdine, and efavirenz. Then, protease inhibitors were introduced

namely ritonavir, saquinavir, and indinavir. HIV-1 protease plays a role in the viral gag and gag-pol polyprotein cleavage during virion maturation (Arts & Hazuda, 2012; Emamzadeh-Fard et al., 2013). These inhibitors were then used in combination with the RT inhibitors and are known as HAART. HAART has the ability to efficiently lower the viral activity and delay progression to AIDS despite its toxicity, side effects and antiviral drug resistance that follow after therapy (Arts & Hazuda, 2012). Integrase inhibitors are another class of antiretrovirals that block the formation of a preintegration complex of viral DNA and host DNA. Raltegravir is one of the approved integrase inhibitors introduced in 2007 followed by elvitegravir. Later, fusion or entry inhibitors were designed and approved clinically. Fuzeon, T20, and maraviroc are included in preventing fusion or entry of the HIV into the cells. The antiretroviral targets are depicted in **Figure 1.1.3**.

Low adherence to HAART or extensive use of antiretroviral drugs among HIV-1 patients is now a major challenge. The patients would slowly develop poor drug tolerability and cross-reactivity among the antiretroviral agents and other medications. This can lead to the evolution of drug resistance and consequently treatment failure. Due to this problem, there is always a pressing need for new HIV-1 treatments (Arts & Hazuda, 2012; Paydary et al., 2013).

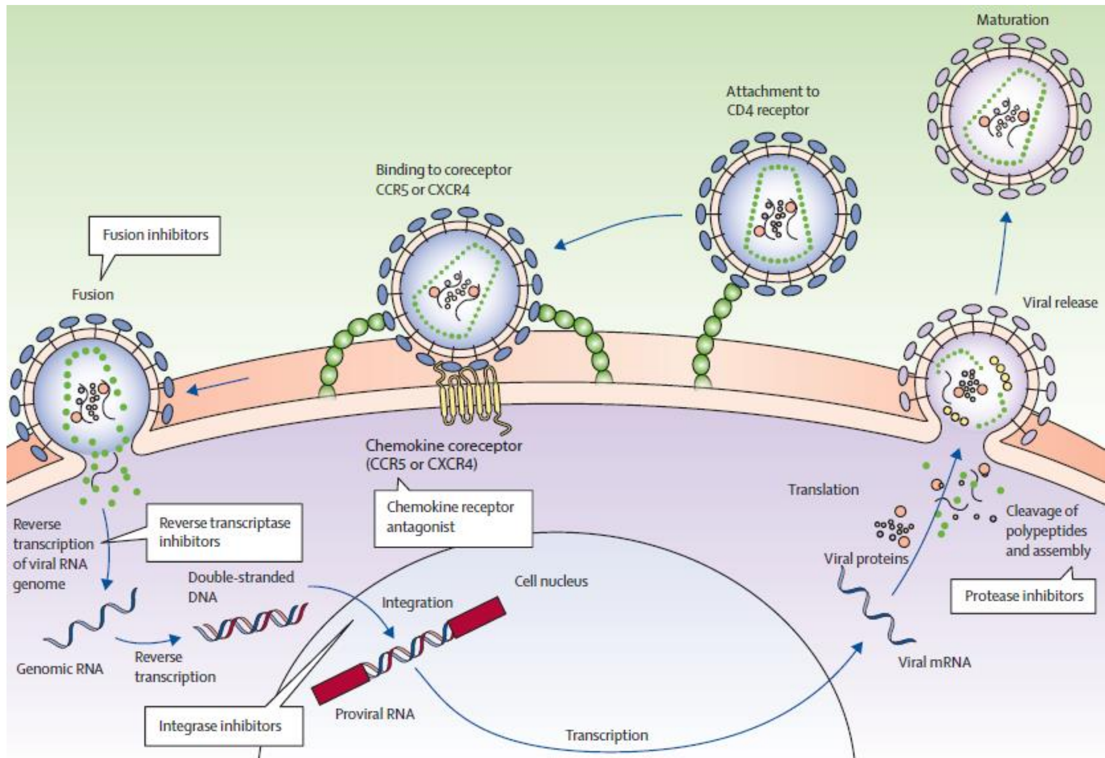


**Figure 1.1.3. HIV life cycle and antiretroviral targets.** Image above shows the sites of action of different classes of antiretroviral drugs (Maartens et al., 2014).

The discovery of antibodies against HIV-1 has increased progressively due to a better understanding of viral structures and mechanism of infection. This is especially against the HIV-1 Env protein which could prevent viral entry. With increasing knowledge on the glycoproteins, many more neutralizing antibodies (NAbs) were able to be developed (Ringe & Bhattacharya, 2013) such as the first broadly neutralizing human monoclonal antibody (mAb) b12. This mAb was found to neutralize clade B viral isolates at the rate of 50%. The Ab targets the CD4 binding site of gp120. It was reported in 1994 that b12 was selected by phage display library method from the bone marrow of HIV-1 infected patient (Barbas & Barbas, 1994). In 1992, mAbs against the P24 protein were produced from hybridomas by Konovalov and group (Konovalov et al., 1992). Their binding activities were examined and the antigenic epitopes of p24 were determined. However, these antibodies were only used to study the antigenic properties of the P24 protein.

HIV-1 CA protein structure consists of two independently folded domains, C-terminus and N-terminus domains, which are connected by a flexible linker (Sticht et al., 2005; Thenin-Houssier & Valente, 2016). The CA protein structure is depicted in **Figure 1.1.4**. Tang et al. (2003) reported that viral particles with unstable capsid due to mutations would severely reduce infectivity. They discovered a potent molecule inhibitor of HIV-1 CA, called CAP-1 (Tang et al., 2003; Kelly et al., 2007; Adamson et al., 2009). This compound was found to inhibit capsid assembly during the maturation step which resulted in impaired virus production.

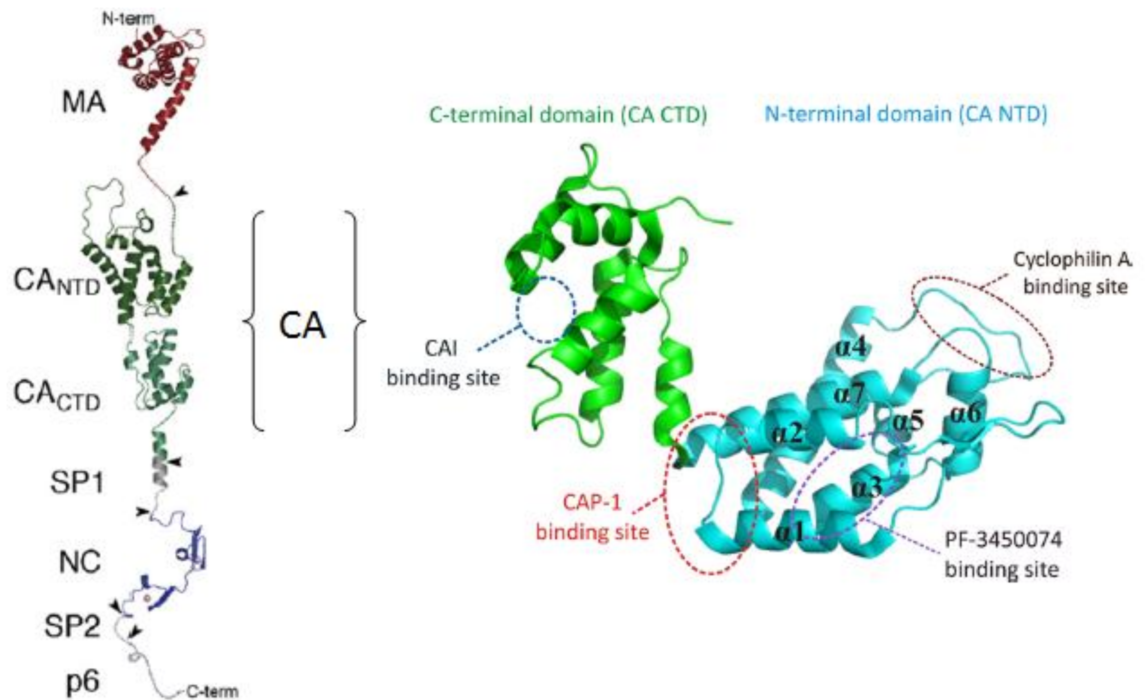


**Figure 1.1.4. Organization and structure of HIV-1 Gag and CA proteins.** The left panel shows a schematic secondary structure of HIV-1 Gag polyprotein. Individual domains are represented in different colors. Protease cleavage sites are indicated by the arrowheads. The right panel shows an illustrative representation of the HIV-1 capsid structure with highlighted ligand binding sites (Machara et al., 2015; Thenin-Houssier & Valente, 2016).
MA (matrix), CA (capsid), NC (nucleocapsid), SP (spacer peptides), and p6 (protein of 6 kDa).

However, it was found not affecting the stability of pre-assembled CA-NC (nucleocapsid) complex in vitro (Fricke et al., 2013). More recent publications reported the details of specific inhibitors of HIV CA assembly by binding to the C-terminal domain (CTD) (Sticht et al., 2005; Bocanegra et al., 2011; Zhang et al., 2011; Marchara et al., 2015; Thenin-Houssier et al., 2016) and N-terminal domain (NTD) (Kortagere et al., 2012; Lemke et al., 2012; Kortagere et al., 2014) of the CA protein. A capsid assembly inhibitor (CAI) was reported to bind the CA-NTD and acted at the late stage of the HIV-1 life cycle (Sticht et al., 2005). However, due to its low cell permeability, it was not suitable for blocking HIV-1 replication in cells. Several years after that, a modified peptide was designed based on the CAI peptide to overcome its limitation. Zhang et al. (2008) successfully produced a cell-penetrating peptide known as NYAD-1 by using hydrocarbon stapling technique. This technique stabilized the peptide to penetrate the cells, but the compound has drawbacks of poor inhibitory effect and short half-life. PF74 is another CA inhibitor that displayed inhibitory activities at the early and late stages of virus life cycle, and can bind to both CA-NTD and -CTD (Blair et al., 2010). Then, a PF74-resistant mutant virus that alters the interaction with the host factors required for viral entry was developed (Zhou et al., 2015). Machara et al. (2015) identified a CA-CTD specific inhibitor, 2-arylquinazolines, which is capable of blocking viral replication. However, it was doubt to have rapid clearance in the cells which thus makes it inefficient. The most recent CA inhibitor is Ebselen (Thenin-Houssier et al., 2016). It was shown to covalently bind to the CA-CTD and inhibit dimerization of the CA at the early stage of HIV-1 life cycle, by impairing the uncoating events. However, it was reported to have low specificity.

The above-mentioned studies had shown that the HIV-1 CA protein is an attractive target for drug development. However, there is still a pressing need to continue the search of therapeutic molecules against the HIV-1 which are more stable, less-resistant, and able to intracellularly inhibit viral infection.

### 1.1.4. Antibody Molecule

Antibodies are produced in response to infections and foreign bodies. They are also known as immunoglobulin (Ig) which is the folding of the amino acid residues in a globular motif. Ig is attached to foreign substances called antigens, to be destroyed by the immune system. Antibodies are synthesized by B cells in two forms, membrane-bound antibodies, and secreted antibodies. This large molecule with molecular weight of ~150 kDa comprises two identical units of heavy- and light-chains which are produced by rearranged germline variable (V), diversity (D) and joining (J) gene segments at the heavy chain locus while V and J gene segments at the light chain locus (Liao et al., 2009). **Figure 1.1.5** shows different antibody structures that are composed of heavy and light chains covalently linked by disulfide bonds. The heavy chain consists of one V region and three or four C (constant) regions. The light chain is composed of one V region and one C region. The V region of heavy chain ($V_H$) and the adjoining V region of the light chain ($V_L$) form the antigen binding domain (Janeway et al., 2001; Hudson & Souriau, 2003; Lo et al., 2008).

There are five classes of antibodies that differ in their heavy-chain, termed as IgG, IgM, IgA, IgD, and IgE. Most of them are distributed or transported to the compartments of the body with appropriate effector functions for each antibody class which are determined by their isotypes (Elgert, 1998; Janeway et al., 2001; Abbas et al., 2015).

IgG contains gamma (ɣ) chain in the heavy chain region and is the most abundant Ig present in serum. IgG subclasses are including IgG1, IgG2, IgG3, and IgG4. It responds directly to toxins and viruses. In HIV, it was reported that IgG3 antibodies are more effective in neutralizing the virus than IgG1 antibodies. IgG is the only

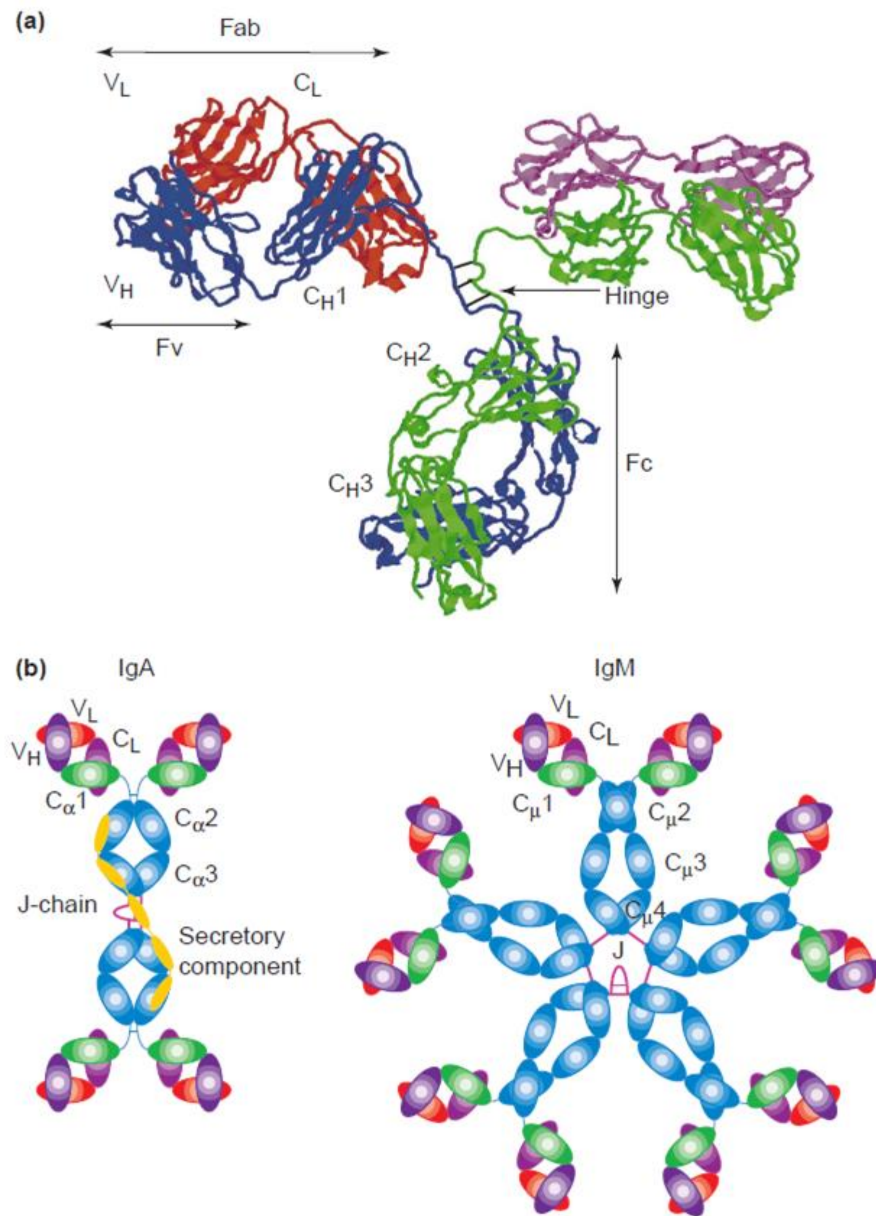


**Figure 1.1.5. Schematic representations of the antibody structure**. **(a)** Domain organization of IgG. Antigen binding area is composed of variable heavy ($V_H$) and light ($V_L$) chains. Constant domains $C_H2$ and $C_H3$ function as the effector component of the antibody for receptor binding. **(b)** The dimeric secretory IgA (SIgA) and pentameric IgM structures. SIgA is a dimer in which monomers are disulfide-linked via J-chain. IgM monomer with a pair of Cμ2 domains replacing the hinge, unpaired Cμ3 domains, and C-terminal tailpieces (Little et al., 2000).

antibody type that is capable of crossing the placenta and is also involved in secondary immune response.

IgM normally exists as a pentamer which contains mu (μ) chain as the heavy chain. It is the first type of antibody expressed on the surface of B cells during an immune response. It provides early fight against pathogens. Its pentameric structure is essential for effective activation of the complement system (Abbas et al., 2015).

IgD is composed of delta (δ) chain in the heavy chain region. It works with IgM in the B cell development (Abbas et al., 2015). However, it circulates at very low levels in the serum with a short serum half-life.

IgE is a monomer type antibody with epsilon (ε) chain. It binds to allergens and is associated with hypersensitivity reactions as well as protects against parasitic worms (Abbas et al., 2015).

IgA is a highly produced Ig that can be found at mucosal surfaces like the gut, respiratory and urogenital tracts. It protects against toxins, virus, and bacteria by neutralization or prevention of binding to the mucosal surface (Abbas et al., 2015). It can also be found in secretions of breast milk as the ‘first milk’ given to the neonate by the mother.

### 1.1.5. Generation of scFv antibodies

The high molecular weight of immunoglobulins (Ig) limit not only their use in high throughput of biochemical and structural studies but also less efficient as biological therapy (Farajnia et al., 2014). There are various types of recombinant antibody formats that have been engineered over the last two decades. Being the most popular one, scFv consists of antibody variable domains connected by a flexible linker and is the mostly used to overcome this drawback (Wörn & Plückthun, 2001).

ScFv is described as a single-chain antibody fragment, a smaller version of the antibody with the complete antibody antigen-binding site. The smallest Ig fragment containing the antigen-binding site is the Fv fragment. It consists of the variable heavy ($V_H$) and –light ($V_L$) chains. The linker between Fv holds higher stability than the Fv itself. It is then recognized as a single-chain Fv (scFv). The length of linker would determine the formation of multimeric forms of the scFv (**Figure 1.1.6**). Linkers can be incorporated in either $V_L$ – linker – $V_H$ or $V_H$ – linker – $V_L$ orientation. Usually, the linker is composed of 15 amino acids with $(Gly_4Ser)_3$. In order to produce scFv, enzymatic cleavage can be done but it is considerably a difficult and less stable process.

ScFvs can be constructed by amplifying the variable regions from mRNA of hybridoma cells using PCR (Toleikis et al., 2004). ScFvs can be produced in *E. coli* using different types of promoters; phage λ, LAC, TAC, or phage T7 (Bird and Walker, 1991). However, there were reports that scFv proteins were usually insoluble in *E. coli* (Sodoyer, 2004), thus they require solubilization and refolding steps. This would be time consuming and laborious. Even with these problems, there were reports of successful scFv expressions

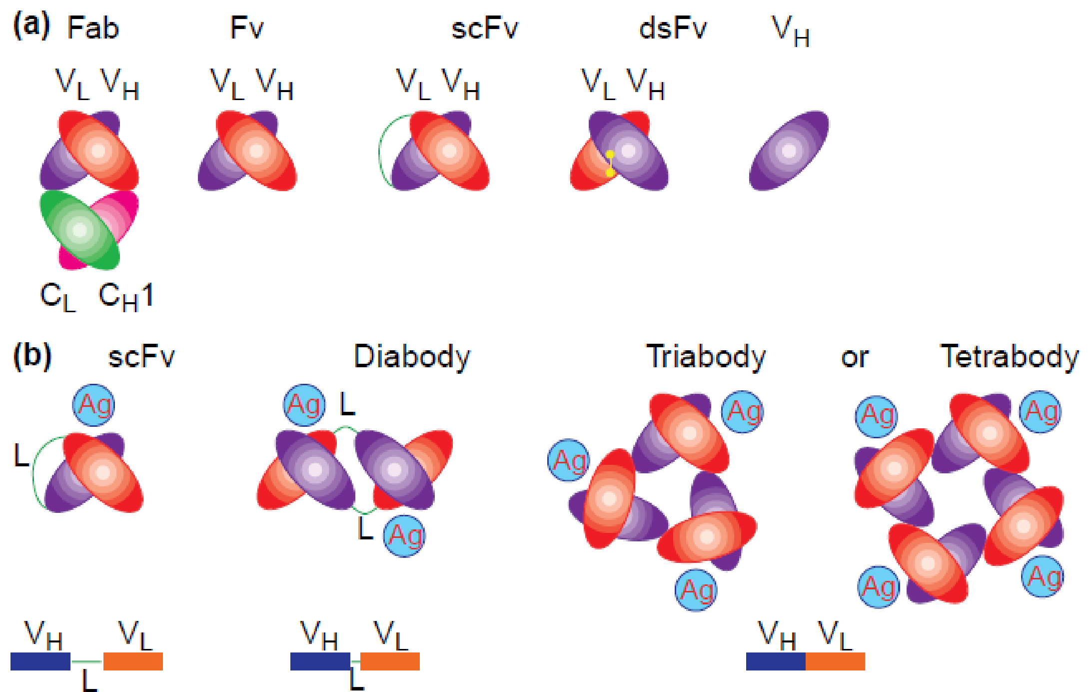


**Figure 1.1.6. Recombinant antibody constructs.** (a) Monovalent fragments of the antibody molecule capable of binding antigen. Fab, Fv, and disulphide-stabilized Fv (dsFv) fragments consist of two separate chains while scFv and single $V_H$ fragments are composed of a single polypeptide. (b) ScFv with a peptide linker that connects $V_H$ and $V_L$ domains, or fused directly without a linker. Different structure formations exist with different length of the linker. Shorten linker may develop a diabody (scFv dimer), triabody (trimer), and tetrabody (tetramer) (Little et al., 2000).

Ag (antigen) and L (linker).

of soluble scFv in *Bacillus subtilis* which did not require the refolding step (Bird and Walker, 1991).

ScFv has become the first option for therapeutic purposes nowadays for having lower retention time in nontarget tissues than Fab. In addition, scFv has more rapid blood clearance and better tumor penetration due to its small size (Bird and Walker, 1991; Kipriyanov et al., 1997; Little et al., 2000; Chadd & Chamow, 2001; Hagemeyer et al., 2009; Farajnia et al., 2014). A previous experiment showed that the use of scFv was better than the larger antibody (Fab) (Bird et al., 1988). There was a study of scFv and Fab being injected into tumor-bearing mice. It was observed that clearance of the scFv was 7 times faster than the Fab molecules. This demonstrated that scFv reaches the tissues and organs faster than the Fab. It could target and localize the tumor tissue better than the Fab (Bird et al. 1988).

Besides, some properties can be easily tailored such as antigen-binding affinity, stability, and expression level of the antibody fragment as compared to the full-length Ig (Mazor et al., 2007). It has become a promising alternative to monoclonal antibodies (Farajnia et al., 2014; Yan et al., 2014). ScFv provides many other applications. Apart from being used as in vivo diagnosis and treatment of diseases, it can be used in biomarker validation (Baird et al., 2010), in vitro diagnosis, biosensors, catalytic antibodies and can be genetically engineered to enhance its functions.

### 1.1.6. Phage display technology

Phage display is a highly powerful technology for producing a large amount of peptides, proteins, and antibodies with novel selection methods to screen the polypeptides with novel functions. This technology applies the physical linkage between phenotype and genotype of the polypeptides that are fused to the bacteriophage coat proteins (Fagerlund et al., 2008; Bazan et al., 2012). The phenotype of the protein is displayed by the bacteriophage while the genotype encoding that molecule is packaged within the same virion (**Figure 1.1.7**). This criterion allows the selection and amplification of specific clones with the desired binding specificity from diverse phage clones. In addition, this technique allows easy determination of the specific binder through DNA sequencing. It was first introduced by George Smith in 1985 and has become an effective tool with applications in the discovery of ligands for affinity chromatography and drugs, in the study of protein/protein interactions, and in epitope mapping (Ehrlich et al., 2000; Fagerlund et al., 2008) thus allowing development of new drugs, vaccines, genetic mapping, and biosensing (Qi et al., 2012).