博士論文（要約）

Identification of macrocyclic peptide-based drug candidates for COVID-19 treatment and development of peptide-based fluorescent tags for specific protein labeling

(COVID-19感染症に資する環状ペプチド薬剤候補およびタンパク質の特異的蛍光化に資するペプチドの開発)

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Abstract

A Peptide which has medium molecular size (2-3 kDa) shows both properties of a small molecule and a protein. With larger interaction surface compared with small molecule, peptide, especially macrocyclic peptide can have high affinity and selectivity to its target and can aim for protein-protein interaction (PPI) in spite of its smaller size than a protein. With these advantages, peptide is regarded as an attractive modality for drug discovery. In addition, peptides are also utilized to functionalize proteins as tags. The peptide tags such as purification tags and fluorescent tags are now essential especially in a bioengineering filed.

We have developed a *de novo* peptide selection method called Random non-standard Peptides Integrated Discovery (RaPID) by the combination of *in vitro* translation system and codon reprograming. We can utilize highly diverse peptide libraries including macrocyclic peptide library for the selection, and peptides with high target affinity and selectivity can be identified.

In this thesis, we performed selections with macrocyclic peptide library by RaPID to identify peptides with suitable activities for each purpose. In the first topic, drug discovery for Coronavirus Disease 2019 (COVID-19) was performed by the selection targeting a spike protein (S-pro) of the virus. The peptides targeting the S-pro has possibility to inhibit the virus infection to human cells. We aimed to obtain peptides with virus infection inhibitory activity.

In the second topic, we tried to develop a new protein imaging method. Inspired by an RNA imaging method, we utilized peptides which activate fluorogenic molecules (fluorogens) as fluorescent tags. A fluorogen shows fluorescence only when its aptamer binds and fixes the structure of the fluorogen to a specific state. Peptide aptamers which activate fluorogens would have potency as small fluorescent tags with minimum background. The tags are expected to make an accurate and clear protein imaging possible.

In chapter 1, general features and applications of a peptide in some research fields are briefly mentioned. Then, the mechanism and procedure of the RaPID is explained.

In chapter 2, RaPID selections targeting S-pro of the virus are described. We identified macrocyclic peptides with high target affinities. Then, evaluations of their bioactivity were performed to identify the drug candidates. The binding site of the peptides to S-pro was considered to estimate the inhibitory mechanisms. Finally, by comparing with bioactivities of identified peptides from selections targeting different partial proteins of S-pro, we considered a suitable target for peptide drug discovery for virus infectious diseases.

In chapter 3, RaPID selection targeting a fluorogen, (4-((2-hydroxyethyl) (methyl)amino)-benzylidene)-cyanophenylacetonitrile (HBC) is described. We first measured HBC activation effects of the identified peptides from the selection. After considerations of suitable structures of

the fluorogens and the peptides for the activity, the peptide sequence was inserted into a model protein to check the potency as a fluorescent tag.

Chapter 4 is the general conclusion of this thesis. It summarizes the achievements of these researches and discusses the perspectives of them.

Contents

Abbreviation list

δ	Chemical shift
A	Adenine
a.u.	Arbitrary unit
Abs.	Abosorbance
Ac	Acetyl
ACE2	Angiotensin-converting enzyme 2
Ala(A)	L-Alanine
Arg(R)	L-Argenine
ARS	Aminoacyl-tRNA synthetase
Asn(N)	L-Asparagine
Asp(D)	L-Aspartic acid
ATP	Adenosine triphosphate
BHQ-1	Black hole quencher 1
bio	Biotinylated
Boc	*tert*-butoxycarbonyl
BSA	Bovine serum albumin
C	Cytosine
Calcd.	Caluclated
CD	Cytoplasmic domain
cDNA	complimental DNA
ClAc	Chloroacetyl
CME	Cyanomethyl ester
COVID-19	Coronavirus disease 2019
CryoEM	Cryogenic Electron Microscopy
Cys(C)	L-Cysteine
d	Doublet
DCM	Dichloromethane
Del	Deletion
DFHBI	
DIPEA	*N,N*-diisopropylethylamine
DMAP	4-Dimethylaminopyridine
DMF	*N*,*N*-Dimethylformamide
DMHBI	3,5-dimethoxy-4-hydroxybenzylidene imidazolinone
DMSO	Dimethylsulfoxide
Dnase	Deoxyribonuclease
dNTP	Deoxynucleoside triphosphate
DTT	Dithiothreitol
E. coli.	Escherichia coli
EDC	1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide
EDT	Ethane dithiol
EDTA	Ethylenediamine-*N*,*N*,*N*',*N*'-tetraacetic acid
EF	Elongation factor
eFx	Enhanced flexizyme
ESI	Electrospray ionization
Et	Ethyl
EtOAc	Ethyl acetate
EUA	Emergency use authorization
FAP	Fluorogen activating protein
FIT	Flexible in vitro translation
Fluorogen	Fluorogenic molecules
fMet	formyl L-methionine
FP	Fusion peptide
FXa	Factor Xa
G	Guanine
GFP	Green fluorescent protein
Gln(Q)	L-Glutamine
Glu(E)	L-Glutamic acid
Gly(G)	L-Glysine
GMP	Guanosine monophosphate
h	Hour(s)
hAGT	human alkylguanine-DNA alkyltransferase
HBC	(4-((2-hydroxyethyl) (methyl)amino)-benzylidene)-cyanophenylacetonitrile
HBTU	2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His(H)	L-Histidine
HOBt	1-hydroxybenzotriazole
HPLC	High Performance Liquid Chromatography
HR	Heptat repeat domain
IF	Initiation factor
IL-6	interleukin-6
Ile(I)	L-Isoleucine
IPTG	Isopropyl *β*-D-1-thiogalactopyranoside
K	Guanine or Thymine (Uracil)
ka	assciation constant
K_D	Equilibrium dissociation constant
kd	dissciation constant
LB	Luria-Bertani
Leu(L)	L-Leucine
Lys(K)	L-Lysine
M	Mol/L
m	Multiplet
MALDI	Matrix Assisted Laser Desorption/Ionization
MBP	Maltose-binding protein
Me	Methyl
MeCN	Acetonitrile
MeOH	Methanol
Met(M)	L-Methionine
min	Mimute(s)
mRNA	Messenger RNA
MS	Mass spectrometry
MTF	Methionyl-tRNA transformylase
N	one of any nucleic acid bases
N	Normality
NFV	Nelfinavir
NHS	*N*-hydroxy succinimide
NMP	*N*-methyl pyrrolidone
NMP	N-methylpyrrolidone
NMR	Nuclear magnetic resonance
NTD	N-terminus domain
NTP	Nucleoside triphosphate
Obsd.	Observed
OD	Optical Density
ODS	Octadecyl silane
P/N	Positive/ negative
PAGE	Poly-Acrylamide Gel Electrophoresis
Pbf	2,2,4,6,7-Pentamethyldihydrobenzofuran-5-sulfonyl
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline (-) with Tween20
PBS-T	Tris-buffered saline (-) with Tween20
PCR	Polymerase chain reaction
PEG	polyethylene glycol
Phe(F)	L-Phenylalanine
PPI	Protein-protein interaction
Pro(P)	L-Proline
Pu	Puromycin
qPCR	Quantitative polymerase chain reaction
r.t.	Room temperature
RaPID	Random non-standard Peptides Integrated Discovery
RBD	Receptor-binding domain
RNase	Ribonuclease

rpm	Rotations per minute
RS	tRNA synthetase
RT	Reverse transcription
Rtase	Reverse transcriptase
RU	Response unit
s	Singlet
SARS-CoV-2	Severe Acute Respiratory Syndrome Related Coronavirus-2
SDS	Sodium dodecyl sulfate
sec	second
Ser(S)	L-Serine
SPC18	Hexa-(ethylene glycol) phosphate
SPR	Surface plasmon resonance
S-pro	Spike protein
T	Thymine
t	Triplet
TBS	Tris-buffered saline
tBu	*tert*-Butyl
TFA	Trifluoroacetic acid
THF	Tetrahydro furane
Thr(T)	L-Threonine
TM	Transmembrane domain
TMPRSS2	Transmembrane protease, serine 2
TOF	Time of flight
Tris	Tris(hydroxymethyl)aminomethane
tRNA	TransferRNA
Trp(W)	L-Tryptophane
Trt	Trityl
Tyr(Y)	L-Tyrosine
U	Uracil
U	Unit
Val(V)	L-Valine
w	D-Tryptophane
X	Arbitrarily amino acid

Chapter 1 General introduction

1.1 Peptide

1.1.1 Property of peptide

A peptide which is composed of several amino acids has high diversity and secondary structure which results in its function. Peptide sequences in nature mainly consist of twenty natural amino acids, whose combination generates the diversity. Moreover, peptide forms a steric structure which characterizes its function. Because of both properties, a peptide can exhibit various functions. Therefore, peptides are attractive for the application in many fields[1–3].

The comparison of a peptide features with that of a small molecule and a protein is shown in table 1-1[4–6]. Because a peptide is medium size (500-3000 Da in general) between a small molecule and a protein, the peptide has advantages of both small molecules and proteins. With large interaction surface to its target, a peptide shows high selectivity and affinity to the target like a protein. On the other hand, a peptide can be prepared by organic synthesis like small molecules, which lead to lower manufacturing cost in commercial use. Moreover, peptides can be easily chemically modified to modulate its property. For example, oligomerization would enhance its activity, and lipidation and polyethylene glycolylation would improve its physical properties[7–10].

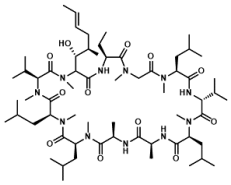
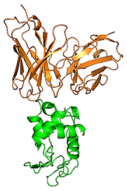
Classification	Small molecule	Peptide	Protein
Structure			
Example	Acetyl salicylic acid	Cyclosporine	Antibody
Molecular weight	<500	500~3000	3000<
Interaction surface	Small	Medium~Large	Large
Target affinity	Low	High	High
Manufacturing cost	Low	Low~Medium	High
Site specific modification	Easy	Easy	Difficult

Table 1-1 Classification of molecules and their properties.

With the advantages of a peptide, it is well applied to pharmaceutical drug[1,4,11,12]. Because of its high target affinity, a peptide often affects to an activity of its target protein. This feature can be used to control life activity and treat diseases. High target selectivity of a peptides is suitable to reduce adverse effects for treatment.

Peptides are also applied for protein engineering as a peptide tag[15,16]. A peptide can be easily inserted into protein structure by gene modification and the function of the peptide can be added to the original protein. Its specific affinity to the target can be utilized for purification of recombinant proteins[17,18]. The function of peptides derived from unique structures, for example such as membrane permeability[19] and solubility[16], can also be imparted to proteins. Peptide tags are now widely applied and is essential for the bioengineering field.

A peptide which is consisted of only natural amino acids can be synthesized by ribosomal translation. This feature is suitable for *de novo* selection to identify peptides which have desired activities (details are described in 1.2.1). On the other hand, the peptide usually has low biostability and low oral availability, which are not suitable for a drug candidate.

To improve these properties, incorporation of unnatural amino acid is a powerful way. For example, incorporation of D-amino acids enhances the resistance to proteases, and incorporation of *N*-alkyl amino acids improves membrane permeability of a peptide. Although usage of unnatural amino acids is effective for drug candidates, application for *de novo* selection is difficult because unnatural amino acids cannot be dealt with by ribosomal translation in general. In addition, application for a peptide tag would be also difficult with the same reason.

Another way to improve properties for a drug candidate is a cyclization. A cyclic peptide usually shows higher biostability than a linear peptide because a cyclic peptide less reactive to proteases. Moreover, a cyclic peptide has higher target affinity than that of a linear peptide because of its rigid structure. This feature is suitable for both a drug candidate and a peptide tag. A macrocyclic peptide library can be applied for *de novo* selection by chemical modification peptides synthesized by ribosomal translation. This means macrocyclic peptides which has desired activity can be easily identified. With the technology named lasso-grafting developed in our laboratory, a macrocyclic peptide can be applied for a peptide tag (details are mentioned in 1.1.3). Because of these reasons, a macrocyclic peptide is a highly potent molecule for applications in various fields.

Historically, it was difficult to discover peptides that had specific function because of the difficulty in preparing peptide libraries for peptide screening. Because of this reason, most of the peptides practically applied were derived from nature. The applications of peptides were limited because of the challenges.

With the development of combinatorial chemistry in the 1990s, construction of peptide libraries became easier, and peptide selections have been performed to identify peptides with desired activity[21,22]. Improvements of peptide selection methods also have led to increase the applications of peptides in various research fields[23].

1.1.2 Application for drugs

Originally, a peptide is one of the biological molecules that regulates biological phenomenon[24,25]. Therefore, the peptide has aptitudes for treatment of diseases. In fact, a number of peptides that have biological activities from natural sources have been applied for therapies. For example, insulin, which is a hormone that regulates blood glucose levels, is used for treatments of diabetes[26]. Most of the peptide drugs emerging in clinical trial were natural peptides and its analogues before the 2000s[1]. Recently, heterologous peptides which is not derived from nature, are emerging because of the developments of peptide screening methods. The number of clinically applied peptides is gradually increasing[1,25] (Table 1-1).

Advantages of peptides as pharmaceutical drugs are high target affinity and selectivity as mentioned in section 1.1.1. Higher affinity often leads to stronger biological activity, and higher selectivity decreases off-target effects. Large interaction surface also enables to access target molecules that have no binding pocket which is difficult to target by small molecules. Protein-protein interaction (PPI), which has important rolls in biological phenomenon, can be easily targeted by peptide too[27,28].

In addition to the positive properties common with proteins, a peptide has possibility for membrane permeability and oral administration like small molecules[29,30]. Cyclosporine, clinically used as an immune suppressor, inhibits cyclophilin, an intracellular protein, and it is orally available[31]. With these attractive properties, peptides are regarded as a new modality and peptide drug discovery has been actively performed.

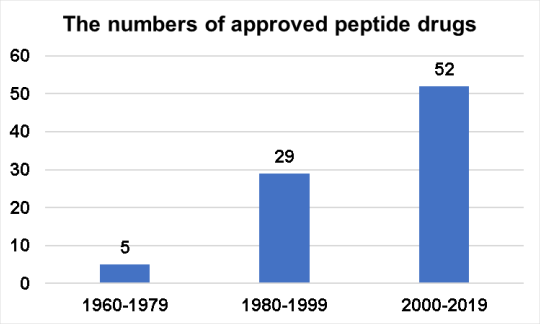


Figure 1-1 The increasing number of peptide drug approval cases.

1.1.3 Application for protein engineering

Peptides can also be applied for functionalization of recombinant proteins. Peptide sequences can be genetically inserted into proteins and fused proteins are expressed by protein expression system with the corresponding genes. Proteins can obtain additional activities by the fusion of peptides, which activities help the proteins for expression, purification modification and so on (table 1-3)[15,16,18].

Peptide tags are often utilized as purification tags[18]. Poly His tag can be fused with N or C terminus of protein and the fused protein obtains affinity to metal ion like nickel[32]. So, His-tagged proteins can be purified by Ni conjugated resin. This procedure helps to selectively purify recombinant protein much easier. FLAG tag and HA tag are also often applied as purification tags[33,34]. Therefore, purification tags are frequently applied for expression of recombinant proteins.

Peptide tags can be reactive positions for site specific modification[3,15,35]. π-clamp, which is composed of only 8 amino acids, can selectively react with perfluoroaromatic ligands to attach to functional groups such as biotin and fluorescent molecules[36]. Site specific modification can functionalize proteins by regulating their properties. This technology is quite important especially for manufacturing medicines such as antibody-drug conjugates[37].

Peptide tags can grant cell permeability to protein[19]. Cell-penetrating peptides like Octa-Arg and TAT peptides have positive charges, and the peptides are cell permeable. Proteins become accessible to inside of cells by the insertion of these peptides, and proteins can target intracellular molecules. The tags are expected as drug delivery tools.

As described above, peptides are suitable for manipulating and functionalizing proteins. By the discoveries of peptides with new activities, the functions can be added to proteins.

Class	Example	Sequence	function or usage	target
Purification tag	Poly His tag	HHHHHH	Affinity purification of recombinant protein	Metal ions like Ni^{2+} or Co^{2+}
	FLAG tag	DYKDDDDK		Anti-FLAG tag antibody
Fluorescent tag	FlAsH	CCPGCC	Fluorescent labeling of fused protein	FLASH-EDT2
	RlAsH	SSPGSS		
Site specific modificataion	π-clamp	FCPW	Site selective chemical modification	Perfluoroaromatic ligand
	CBT tag	VTNQECCSIPM		CBT ligand
Cell penetrating peptide	TAT peptide	GRKKRRQRRRPQ	Interalization of protein into cell	Cell membrane
	Poly Arg tag	RRRRRRRR		

Table 1-2. Representative peptide tags and their usage.

Most of peptide tags are linear form because they can be easily fused to proteins. It is rarely reported that a macrocyclic peptide is applied for a peptide tag because it is difficult to insert a macrocyclic peptide to protein by keeping the structure. Recently, we developed the technique named lasso-grafting[20]. The technique is to insert a macrocyclic peptide sequence into a loop region of a protein by genetic editing without losing both macrocyclic peptide and protein. With the lasso-grafting, not only a linear peptide but also a macrocyclic peptide can be applied for a peptide tag.

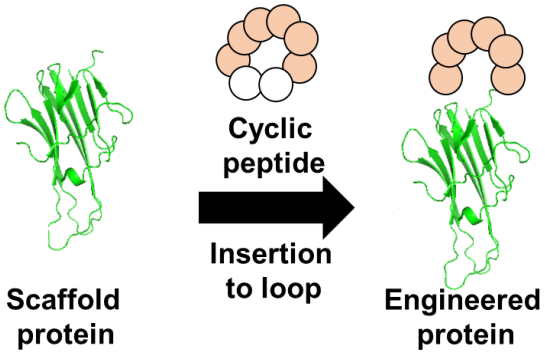


Figure 1-2 Lasso grafting technology.

1.2 *De novo* selection system of peptide

1.2.1 Examples of peptide selection methods

Representative examples of peptide selection methods are shown in table 1-3[23]. In the 1990s, peptide selection methods emerged with the development of combinatorial chemistry[22]. one-bead one-peptide was one of the oldest pooled screening technologies developed by Lam *et al*[21]. The peptide library was constructed by solid-phase peptide synthesis (SPPS) with split and pool technique in this method. Because unnatural amino acids are easily induced in this method, the peptide library can be easily specialized for preferable properties such as hydrophobicity, and biostability. However, diversity of the library is limited to 10^6 because it requires many efforts to expand the diversity further.

Application of ribosomal translation system expanded the library diversities. Phage display is a peptide selection method which utilizes peptide libraries prepared by *in cellulo* translation[38,39]. Phage DNA library encoding various peptide sequences is transfected to its host cells to construct phage library displaying peptide sequences on the surface of phages. By collecting phages which bind to selection targets and read the DNA sequences, peptides with affinity to the targets can be identified. With ribosomal translation, which is rapidness and one-pot peptide synthesis, the library can be diverse to 10^9 in this method. Although the preferable features, only natural amino acids can be incorporated into the peptide library in ribosomal translation in general. Therefore, application of unnatural amino acids is limited.

mRNA display is other method using *in vitro* ribosomal translation[40]. Conditions of *in vitro* translation can be easily modified according to selection purpose, comparing with *in cellulo* translation. This feature is preferable for introduction of unnatural amino acids.

In our laboratory, a new peptide selection method called Random non-standard Peptides Integrated Discovery (RaPID)[41], was established by combining of the mRNA display and codon reprograming (details are described from the next section). Codon reprograming is a technique to change codon-amino acid combinations from original combinations in natural codon tables[42]. This technique enables us to incorporate unnatural amino acids in ribosomal translation. Therefore, unnatural amino acid can be applied to the peptide library while keeping its high diversity in RaPID system[43]. Selection with cyclic peptide library is also accessible with RaPID, and a number of macrocyclic peptides which shows high target affinity has been identified[44–46].

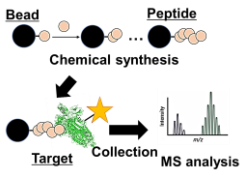
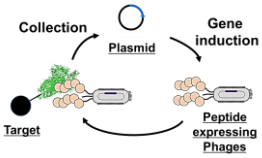
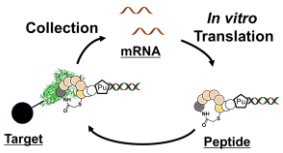
Method	One-bead one-peptide	Phage display	RaPID（mRNA display）
Image			
Synthesis method	Combinatrial synthesis	*in cellulo* translation	*in vitro* translation
Library diversity	10^6	10^9	10^{13}
Peptide detection	MS analysis	DNA sequencing	DNA sequencing
Unnatural amino acids	Available	Partially available	Available

Table 1-3 Representative affinity-based peptide selection methods.

1.3 RaPID system for selection of functional peptides

1.3.1 Flexizyme for aminoacylation and codon reprograming

Codon reprograming in RaPID system is briefly described in this section. In ribosomal protein/peptide synthesis, aminoacylated tRNAs, with anticodon corresponds to sequence of mRNA, are introduced into the ribosome and the amino acids are incorporated into peptide sequences. Aminoacylation of tRNA is carried out by aminoacyl-tRNA synthetase (ARS), and the combination of tRNA and ARS is strictly determined. This feature contributes to the accuracy of ribosomal peptide synthesis, and it is also a cause of difficulty in incorporation of unnatural amino acids.

To apply unnatural amino acids for translation, our laboratory previously developed artificial ribozyme called flexizyme which can catalyze tRNA acylation reaction with various amino acids[47,48]. By the flexizyme, tRNAs can be aminoacylated with amino acids whose carboxylic acids are activated *in vitro*. Although ARS recognizes the side chain of amino acids, flexizyme recognizes only the activated ester moiety[49]. Therefore, various amino acids including unnatural amino acids can be dealt with flexizyme.

Unnatural amino acids can be incorporated into peptides by the combination of flexizyme and reconstitute translation system, called flexible *in vitro* translation (FIT) system (figure 1-3)[50]. Amino acids of interest are reacted with tRNAs by the flexizyme. Then, arbitral amino acids are removed and the aminoacyl tRNAs which have anticodon corresponding the removed amino acids are added into *in vitro* translation system. This procedure makes the codons vacant and assigns any amino acids to the codons. With this technique, peptide library with unnatural amino acids can be constructed.

Macrocyclic peptide library can be also constructed with the FIT system[51]. By incorporating *N*-cloroacetylated amino acid to the initiation codon instead of formyl methionine, peptides with chloroacetyl group (ClAc) at its *N*-terminus are synthesized by translation. Cysteine in the peptide sequence reacts with the ClAc to form thioether bond in the peptide structure (figure 1-3a).

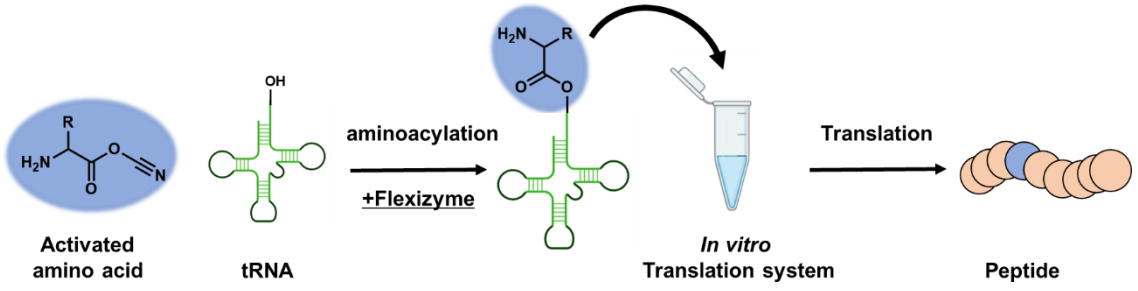


Figure 1-3 Incorporation of optical amino acids into peptide by FIT system.

1.3.2 Scheme of RaPID system

RaPID is the mRNA display selection method with the peptide library constructed by the FIT system[41,43]. Because the restriction of library diversity is the number of ribosomes in translation mixture, it can be expanded to 10^{13} diversity. This diversity leads to the identification of highly potential peptides which shows high affinity and selectivity to their targets. Macrocyclic peptides have especially higher target affinity and selectivity than linear peptide because of its rigid structure[13]. Therefore, selections with macrocyclic peptide library are often performed by RaPID and a number of peptides which shows high binding affinity and biological activity have been identified.

Procedure of RaPID selection is shown in figure 1-4. mRNA library which contains random region, $(NNK)_n$ codon, located between the initiation codon (AUG) and cysteine codon (UGC), is used for the selection. mRNA library is ligated with puromycin linker and *in vitro* translation is performed to generate mRNA-peptide complexes. Assigning *N*-chloroacetylated amino acid to initiation codon by FIT system, allows cysteine after the random region to react with the ClAc to form macrocyclic peptides. Macrocyclic peptide-cDNA complexes are formed by reverse transcription. The complexes are first incubated with negative beads which is not coated with target protein to remove nonspecific binder. Then, the peptides are incubated with positive beads which are coated with target proteins and bound peptides are recovered. The recovered cDNAs are amplified and transcribed to obtain mRNAs. To repeat the cycles, only the peptide sequences with high target affinity are converged. By sequencing the recovered DNA, hit peptide sequences are identified.

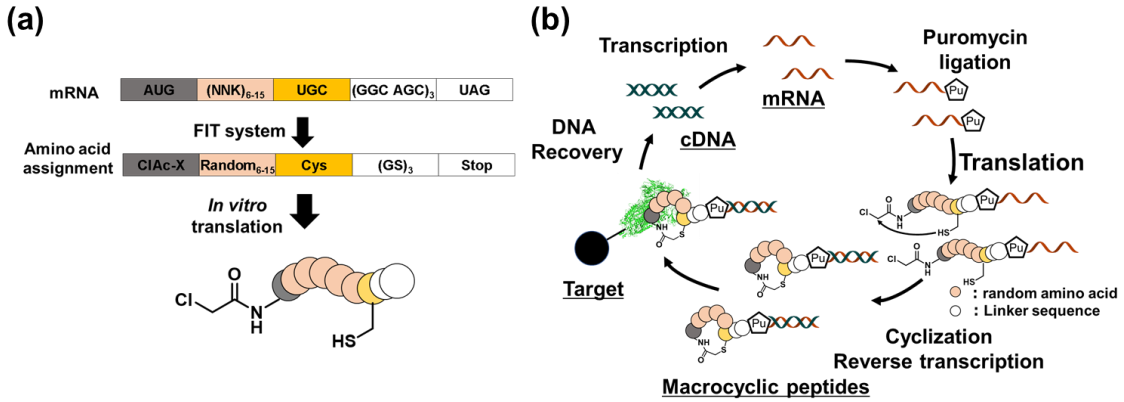


Figure 1-4 (a) Codon reprograming and *in vitro* translation for macrocyclic peptide library. (b) Scheme of RaPID selection with macrocyclic peptide library.

1.4 Purpose of this study

Here, we performed selection with macrocyclic peptide library by RaPID to identify peptides with suitable activities for each purpose.

In chapter 2, we aimed to discover drug candidates for an infectious disease. We performed selection to a virus spike protein and identified peptides which inhibit activity of the spike protein and suppress the viral infection.

In chapter 3, we aimed to develop a peptide-based fluorescent tag for protein imaging. We performed a selection to fluorogenic small molecule and identified a peptide which activate the molecules. The peptide showed potency for the fluorescent tag.

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Chapter 2 Identification of macrocyclic peptide-based drug candidates for COVID-19 treatment

2.1 Introduction

2.1.1 SARS-CoV-2 and COVID-19

Coronavirus disease 2019 (COVID-19), induced by Severe Acute Respiratory Syndrome related CoronaVirus-2 (SARS-CoV-2) is highly infectious and fatal, which is causing the ongoing pandemic[1]. The first case was observed at the end of 2019 and rapidly spread to whole world to be declared as pandemic by World Health Organization in March 2020. As of October 2021, total infection cases are over 240 million and total death reached 4.8 million all over the world (figure2-1) according to the aggregation by Johns Hopkins University. The patients have high fever and feel fatigue in the early stages[2]. The virus replications rapidly proceed, and acute respiratory distress syndrome and acute cardiac injury were induced. In severe cases, the function of lung was destroyed, and the patients reach death. In addition, some patients have sequelae such as fatigue and breathlessness even after they are recovered[3]. This pandemic gives pressure to medical services and the economy which prevents us to sustain our normal lives[4]. Establishment of effective COVID-19 treatments is required to overcome the pandemic[5–8].

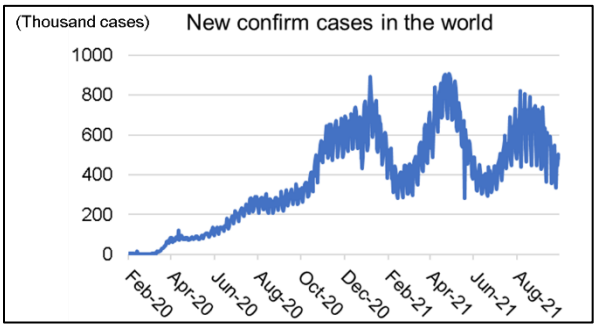


Figure 2-1 COVID-19 infection cases in the world.

2.1.2 Infection mechanism of SARS-CoV-2

The infection mechanism of SARS-CoV-2 is shown in figure 2-2[9,10]. First, pre-fusion form of spike protein (S-pro) on the surface of SARS-CoV-2 binds to angiotensin-converting enzyme 2 (ACE2) on the surface of human cells. Then, protease on the surface of human cells, furin and transmembrane protease, serine 2 (TMPRSS2) cleave the furin cleavage site to remove the S1 domain of S-pro. This cleavage promotes drastic conformational change from pre-fusion to post-fusion form. The S-pro just after the cleavage is named as 'Pre/Post-fusion intermediate'. Appearing S2 domain of S-pro interacts with human cell membrane and membrane fusion is induced. Finally, the virus fuses with human cell and viral RNA enter the cell and virus replication occurs. The replicated viruses infect other cells again. Repeating this cycle, viruses explosively spread to whole human body.

As the interaction between S-pro and ACE2 is essential for virus infection, this PPI is the attractive target for infection inhibition. In fact, neutralizing antibodies targeting S-pro have been reported and REGEN-COV which is the mixture of two neutralization antibodies was issued emergency use authorization (EUA) by the Food and Drug Administration. REGEN-COV is now clinically used for COVID-19 treatment[11].

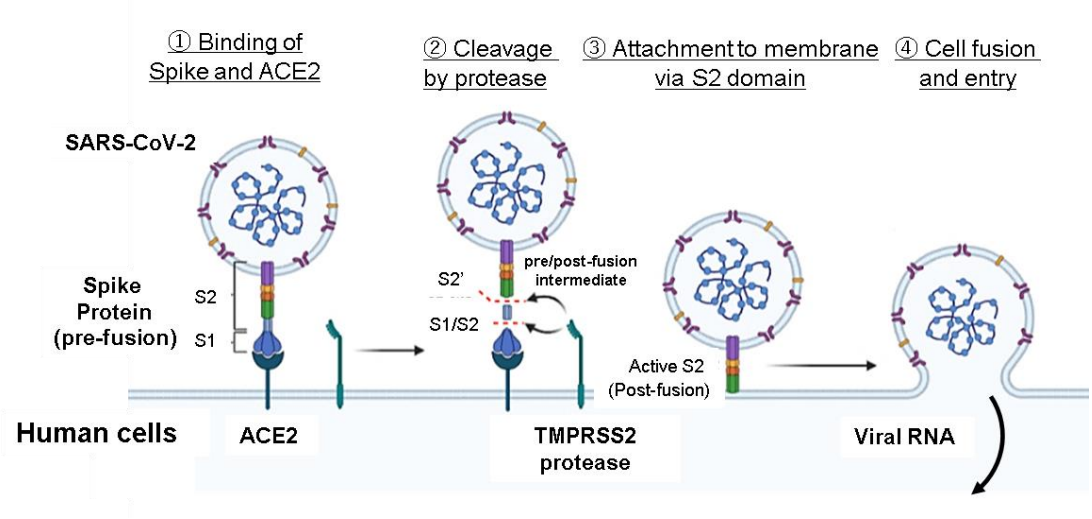
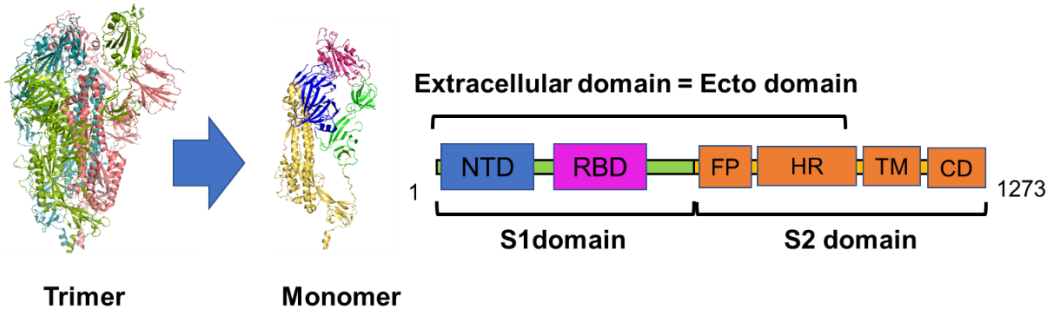


Figure 2-2 Infection mechanism of SARS-CoV-2.

2.1.3 Structure of SARS-CoV-2 spike proteins

SARS-CoV-2 S-pro forms a homotrimer in nature (figure 2-3)[10,12,13]. The monomer is composed of S1 domain and S2 domain. S1 domain mainly contributes to the binding of ACE2, and it is also composed of N-terminus domain (NTD) and receptor-binding domain (RBD). NTD determines host range and RBD directly binds to ACE2. S2 domain mainly contributes for membrane fusion and trimer formation. S2 domain contains fusion peptide (FP) for induction of membrane fusion, two heptat repeat domains (HR) for membrane fusion, transmembrane domain (TM) and cytoplasmic domain (CD) for trimer formation. The furin cleavage site is between S1 and S2 domain. Extracellular domain which contains whole structure without TM and CD is named as ectodomain.

RBD is regarded as a promising target to inhibit PPI between S-pro and ACE2 because RBD directly interacts with ACE2. In fact, a number of S-pro neutralization antibodies including casirivimab and imdevimab, the active ingredients of REGEN-COV, were obtained by applying RBD as an epitope[14].



Large domain	Domain name	Position	Functions
S1 (1-685)	N-terminus domain (NTD)	13-305	Define host species
	Receptor binding domain (RBD)	319-541	Interact with ACE2 directly
	Furin cleavage site	682-685	Have cleavage sequence for furin
S2 (686-1273)	Fusion protein (FP)	788-806	Initiate membrane fusion
	Heptad-repeat domains (HR)	912-1212	Induce membrane fusion
	Transmembrane domain (TM)	1213-1236	Interact with viurs membrane
	Cytoplasmic domain (CD)	1237-1273	Contribute to form trimer

Figure 2-3 Structure of SARS-CoV-2 spike protein.

2.1.4 Reports of developments of SARS-CoV-2 infection inhibitors

COVID-19 therapies which have been applied in clinical treatment are listed in Table 2-1. Vaccines for SARS-CoV-2 are effective to prevent the virus infection, some of which have reported prevention efficiencies over 90%[5,15]. However, the effects are still limited and emerging of mutant viruses may decrease the effects. Therefore, establishments of therapeutics for COVID-19 are of great interest[16].

The first medicine was remdesivir, which was issued EUA in May 2020[17]. Remdesivir is a nucleic acid derivative and inhibits the viral RNA replication. Although remdesivir is effective to various RNA viruses and is used for severe patients to mitigate their symptoms of COVID-19, severe adverse effects are problematic and cannot be applied to mild patients or for infection prevention. Target selectivity is an important factor to reduce adverse effects.

The next medicine issued EUA is tocilizumab, interleukin-6 (IL-6) receptor inhibitory antibody[18]. Although tocilizumab is effective to suppress cytokine storm which is excess inflammation reaction to the virus, it is a symptomatic treatment and virus infection itself can never be inhibited.

In November 2020, REGEN-COV, which is the mixture of two neutralization antibodies targeting S-pro, was issued EUA[11]. These antibodies inhibit the interaction between ACE2 and S-pro to prevent virus infection. REGEN-COV can treat to mild patients to avoid from getting severe. By the combination of antibodies with different binding mode, REGEN-COV keeps treatment efficiency to SARS-CoV-2 mutants. This fact indicates that variety of inhibitors which has different binding mode is highly important to sustain the therapeutic efficiency[19].

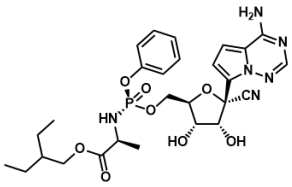
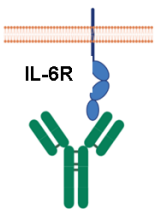
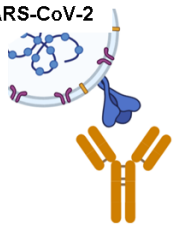
Class	Anti-virus drug	Immune suppressor	SARS-CoV-2infection inhibitor
Image			
Drug name	Remdesiver	tocilizumab	REGEN-COV
Compound	Nucleic acid derivative	Anti-IL-6 receptor antibody	Anti-Spike protein antibodies
Mechanism	Inhibition of virus RNA synthesis	Suppression of immune response	Inhibition of spike protein
Application	Treatment for severe patients	Suppression of cytokine storm	Suppression of becoming severe
Hardship	Possibility of severe adverse effect	No effect for virus infection	Possibility to decrease effects for mutatnts

Table 2-1 Medicines for COVID-19 in clinical use.

2.1.5 Aims of this study

In this research, we tried to identify macrocyclic peptides with SARS-CoV-2 infection inhibitory activity. As of April 2020, when we started this research, there were no therapies for COVID-19. Thus, it was so important to create drug candidates for COVID-19. At the present, there are no peptides that inhibit the virus infection. So, this research still has importance to have various modalities and inhibition mechanisms to oppose the virus mutants.

Macrocyclic peptide is a suitable modality to target extracellular PPI[20]. As mentioned in chapter 1, peptides can target and inhibit PPIs. In addition, manufacturing cost is lower than that of antibodies. Moreover, peptides have the possibility to show different binding mode from antibodies because of its compact structure. These features are good advantages for the purpose of the research.

In addition to the purpose above, we tried to obtain the knowledge about the suitable target protein for peptide selection. Targeting RBD is expected to obtain infection inhibitors effectively because RBD is the position that directly interacts to ACE2. In fact, many neutralization antibodies were identified by targeting RBD[6,21–23]. On the other hand, targeting full length S-pro may leads to discover infection inhibitors with unique inhibition mechanisms because of the complex mechanisms of virus infection[9]. There are no reports mentioning the suitability for peptide selection target. Therefore, acquisition of the knowledge is meaningful for quick responses toward the coming new infection diseases in the future.

本章の以降の内容については、

5 年以内に雑誌等に刊行予定のため、非公表

Chapter 3 Development of peptide-based fluorescent tags for specific protein labeling

3.1 Introduction

3.1.1 Protein imaging

Thousands of various proteins are interacting with one another complexly and the networks make up life activities[1–3]. Therefore, understanding of the interactions and functions of proteins are essential to identify how lives work and how diseases emerge. These elucidation leads to the investments of new biotechnologies and establishments of disease treatments.

Protein imaging is a powerful method to observe dynamics of proteins of interest directly in cells[4,5]. Localization and behavior of specific proteins can be seen by imaging, and it gives a precious information to elucidate protein functions and cellular processes. A fluorescent labeling is frequently applied for protein imaging because it is highly sensitive and selective. Several proteins can be imaged at the same time by using fluorescent molecules with different fluorescent colors. The multicolor imaging is frequently applied to detect co-localization of molecules and visualization of organelles in biological researches[6].

3.1.2 Fluorescent labeling methods for protein imaging

Common fluorescent labeling methods are shown in table 3-1[4,7]. Fusion of fluorescent protein is one of the major methods[8–10]. A fluorescent protein has a fluorescent core in the structure and shows bright fluorescence. Green fluorescent protein (GFP), isolated from *Aequorea victoria* was first identified in 1962[11], and fluorescent proteins with various fluorescent colors have been developed. A fluorescent protein can be fused with a protein of interest by gene editing. For the selective labeling and easy procedure, fluorescent proteins are widely applied for protein imaging. However, fluorescent proteins are so large (*e. g.* GFP is 27 kDa) that they sometimes affect to the behaviors of labeled proteins. This feature prevents observing the original behaviors of proteins.

To overcome the unpreferable features of fluorescent proteins, fluorescent tags have been developed[12–16]. A fluorescent tag is the moiety which can be attached with fluorescent molecule selectively. One of the advantages of a fluorescent tag is that protein can be labeled by smaller moiety so that the tag is less affective to labeled protein's behavior. Fluorescent tags can be roughly categorized to enzyme-based one and peptide-based one.

An enzymatic-based tag is designed from an intrinsic enzyme which has high substrate selectively. For example, SNAP tag is designed from a human alkylguanine-DNA alkyltransferase (hAGT), which catalyzes guanine methylation[17]. The hAGT recognizes benzyl guanine selectively and forms covalent bond to benzyl moiety of its substrate. SNAP tag is labeled by benzyl guanine conjugated fluorescent molecule with the same manner as hAGT. For high substrate selectivity, an enzyme-based tag shows low background fluorescence. However, the size is still large and perturbative. It is also problematic that its intrinsic enzyme is also labeled by the substrate.

A peptide-based tag is so small that it is less perturbative to a protein of interest[18,19]. However, selectivity to fluorescent molecule is low and background fluorescence is high in general. FlAsH tag is an example of peptide-based tag[15,20]. FlAsH tag consists of only six amino acids and thiol moieties of Cys react with the fluorescent reagent to form coordination bonds via arsenic atoms. Although its small structure is suitable for a fluorescent tag, Cys in other proteins reacts with the reagent to induce high background[15]. Cytotoxicity of the arsenic atoms are also problematic for cell imaging.

Considering these properties of a fluorescent protein and a fluorescent tag, a new fluorescent labeling method which is small and shows low background is desired for accurate and clear protein imaging.

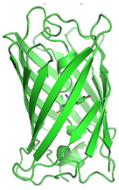
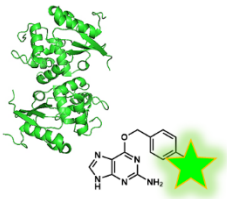
Class	Fluorescent protein	Fluorescent tag	
		Emzyme-base	Peptide-base
Structure			
Example	GFP	SNAP tag	FlAsH tag
Molecular weight	27 kDa	19 kDa	6 a.a.
Advantages	✔ Easy and selective ✔ Low background	✔ High reactivity to its substrate ✔ Low background	✔ Less perturbative
Disadvantages	✘ Affect to protein of interest	✘ Affect to protein of interest ✘ React with endogenous emzyme	✘ High background ✘ Cytotoxicity of As atom

Table 3-1 Fluorescent labeling techniques for protein imaging.

3.1.3 Fluorescent labeling methods for RNA imaging

Fluorescent labeling methods of another bioactive molecule RNA are briefly introduced in this section. RNA-fluorescence *in situ* hybridization was developed in the 1980s and has been widely applied[21]. For an imaging of RNA of interest, its complemental RNA labeled with a fluorescent molecule was utilized to label the RNA of interest by annealing these RNAs. Although the technique can label RNAs in cell, it cannot be applied for live-cell imaging because fixing of cells is required.

Recently, labeling methods for live-cell RNA imaging have been developed[22,23]. Jeremy *et.al*. discovered RNA aptamer named 'Spinach' that binds and activates a fluorogenic molecule (fluorogen) 3,5-dimethoxy-4-hydroxybenzylidene imidazolinone (DMHBI)[24,25]. DMHBI was designed from active center of GFP. DMHBI does not show fluorescence in normal condition but shows fluorescence when DMHBI is fixed to a specific conformation by binding Spinach RNA. Therefore, RNAs fused with spinach sequence can be selectively labeled in living cell in the presence of DMHBI. Wash out of DMHBI is not required because of its minimum background, which is suitable for live cell imaging. Various Aptamer-fluorophore complexes have been developed and many fluorescent colors and multiple labeling are available now[26–29]. (4-((2-hydroxyethyl) (methyl)amino)-benzylidene)-cyanophenylacetonitrile (HBC, **1**) is one of fluorogens, and Pepper RNA-HBC developed by Chen *et. al.* is one examples of the complexes[30,31]. Pepper-HBC complex shows higher brightness and higher signal/background ratio than other RNA-fluorogen aptamer complexes. This feature enables for RNA imaging with high sensitivity.

In addition to fluorogens like DMHBI, fluorescent molecule-quencher conjugate (probe) is also available for aptamer imaging[32,33]. A probe shows no fluorescence because generated fluorescence is absorbed by the quencher. RNA aptamer of a fluorophore or a quencher dissociates the fluorophore-quencher complex in the probe and deactivates the quencher's fluorescent cancellation. RNA aptamer of a sulforhodamine B called SRB, activates the SR-quencher probe and SRB could visualize RNA of interest in live cell[34]. An RNA aptamer of a quencher can also activate a probe. DNB, which is an aptamer of a quencher dinitroaniline, was applied for an RNA imaging tool[35].

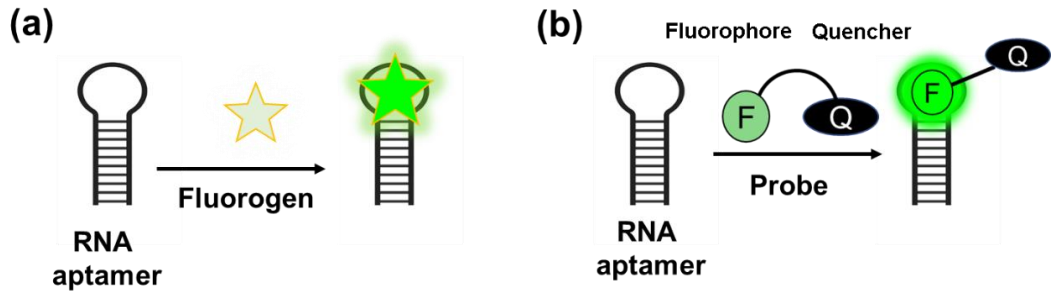


Figure 3-1 RNA aptamer for RNA labeling; (a) Activation of fluorogen. (b) Inactivation of quencher in probe.

These wash-free and low background imaging methods are now widely applied for purchasing behaviors of RNAs of interest in cells. This fact indicates that aptamers of fluorogens or the probes can be utilized for developments of brand-new imaging methods for other biological molecules. These RNA aptamers were identified by Systematic Evolution of Ligands by Exponential Enrichment method targeting fluorogens, fluorophores and quenchers, which is an affinity-based *in vitro* selection method[36].

3.1.4 Aim and strategy of this research

In this research, we aimed to develop a new protein labeling method to overcome problems of current techniques mentioned in 3.1.2. To achieve the goal, we tried to apply the concept of RNA imaging technique to protein imaging. We thought that peptide aptamers which bind and activate fluorogens or probes can be applied to peptide tags with small size and low background.

High target affinity and small size of a peptide are suitable features for the purpose. In general, a peptide rarely has rigid structure enough to bind to small molecules selectively. Therefore, there are few small molecule-binding oligopeptides ever reported. In this research, with highly diversity of peptide library and rigid structure of macrocyclic peptides, we expected that *de novo* peptide aptamers with high target selectivity and affinity can be identified by selections.

Ease of introduction to proteins is also an advantage of a peptide. With the small structure, a peptide not only can be fused to terminuses of a protein, but also can be inserted in the middle of sequence of a protein. Especially, a macrocyclic peptide can be inserted into a loop region of a protein with the lasso-grafting technology mentioned in 1.1.3[37]. These flexible introduction ways would enable peptide tags to be applied various types of proteins.

Moreover, the imaging method would be wash-free by utilizing fluorogenic molecules. Thus, it can be applied for live cell imaging. This tag would be a versatile live cell imaging method which is less perturbational, clearly detectable and easily applicable.

本章の以降の内容については、

5 年以内に雑誌等に刊行予定のため、非公表

Chapter 4 General conclusion

本章の内容については、

5 年以内に雑誌等に刊行予定のため、非公表

List of accomplishment

【Publication】

1.’ Targeting of extracellular protein–protein interactions with macrocyclic peptides’
Shota Taguchi and Hiroaki Suga, *Current opinion in chemical biology*, **2021**, *62*, 82–89.

2.「創薬シーズとしての環状ペプチドの優位性」

田口翔大、西村仁孝、後藤佑樹、加藤敬行、菅裕明、生物工学会誌 第 99 巻 第 4 号 176-179, 2021.

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