Antibody Binding Peptides and its Applications to Antibody Purification and Homogeneous Detection

抗体結合ペプチドの探索と抗体精製及び
ホモジーニアス検出への応用

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Chapter 1

General introduction

1.1. Wide applications of protein binding peptides

Peptide, the molecules consisting of several numbers of amino acids with the molecular weight of 1,000-10,000 dalton, is one of the most significant biological molecules which can regulate the complex biological mechanism in our body. Most of peptides originate from the proteins degraded by proteases and peptidases, and the bioactivity of peptides is induced by binding to various receptors. Over the decades, numerous bioactive peptides such as hormone peptides [1-8] were reported using various methods such as matrix-assisted laser desorption ionization mass spectrometry [9], phage display [10], and solid phase synthesis technology [11]. These peptides have been widely used as effective molecules in the field of pharmaceutics, cosmetics and functional foods, because peptides are highly biocompatible and rarely side effects [12-14].

Recently, peptides have been attracted as protein purification tags, ligands and

probes for target proteins detection because peptides bind to target proteins with high affinity and high stability under various conditions. A number of peptide tags [15-17] and ligands [18-20] have been reported. His tag ($(His)_{6-10}$) [16] and FLAG tag (DYKDDDDK) [17] have been used as purification tags of various expressed proteins. Purification of antibody using epitope and mimotope peptides have been also investigated [18].

Moreover, a number of peptide probes for bio-imaging and diagnostics have been also reported [21-24]. For example, peptide probes for imaging of cancer [21], and peptide probes for detection of biomarker proteins such as Troponin I and T in cardiac diseases [22], and HIV antibodies [23] were reported by many researchers.

Thus, as described above, peptides that bind to various target proteins can apply to various biological research fields, and currently, such peptides are significant as molecules of purification and detection of target proteins.

1.2. Applications of antibody binding peptides to antibody purification

1.2.1 Antibody and issues of antibody production

Antibody is a glycoprotein produced by B cells and is produced naturally as a part of the immune system of mammals by recognizing antigen. The structure of an antibody comprises two antigen-binding domains (Fab's) and a constant region (Fc), involved in effector functions, linked via the flexible hinge region. These regions are part of two pairs of polypeptide chains (with heavy and light chains) folded into compact globular domains. Antibodies bind to target molecules with high specificity and affinity. Thus, since 1890 when antibodies were discovered, antibodies have been attracted as

molecules for medical diagnostics, drug and detection of various targets.

The processes to generate antibodies with affinity and specificity for a target antigen involve some steps such as hybridoma screening, cell culture and purification of antibody. In the processes, large-scale culturing facilities, a lot of expensive culture medium, culture-medium filters and resins for antibody purification are required. The cost of antibody production is 3-10 times higher compared to small-molecule compound [25]. Therefore, the cost reduction of antibody production processes has been an important issue. In order to reduce the cost, various new methods have been received much attention such as the efficient screening methods and culture methods of the antibody producing cells [26, 27], the new ligands for antibody purification [20], the production methods using a low cost host such as *E-coli* [28], yeasts [29], plants [30] and chickens [31].

Although the various approaches to reduce the cost of antibody production, efficient recovery of antibodies from cell culture medium is a critical part of minimizing manufacturing costs. Fig. 1 shows the general rate of the cost in therapeutic antibodies manufacturing process. As shown in the Fig. 1, a significant percentage (40-70%) of the total manufacturing cost of therapeutic antibodies is incurred during purification [32]. Therefore, the cost reduction of antibody purification processes has been most important issues.

1.2.2 Protein A

Protein A is most widely used for purification of antibody from cell culture medium. Protein A is a 56 kDa protein which is originally located in the cell wall *Staphylococcus aureus*, and binds to Fc region of IgG [33]. However, Protein A ligand

suffers from a number of problems, that is, the extremely expense, its contamination risk of biological origin (difficulties in isolation and purification from microbial extracts), the requirement for accurate analytical tests to ensure the absence of toxic contaminants, and the poor stability to cleaning and sanitizing agents. Furthermore, there is potential risk of contamination of Proten A itself in the final product (ligand leakage resulting from harsh elution conditions) [20]. Therefore, peptidic affinity ligands and non-peptidic affinity ligands have attracted great interest as alternatives to Protein A (Table 1).

1.2.3. Peptidic ligand

Over the past decade, the construction of peptide libraries has assumed an important role in the identification and characterization of ligand-receptor interaction. For example, phage display [10] (biological strategy), combinatorial split synthesis [34] (chemical strategy) and beads display [35] (chemical strategy) are most popular and classical method of synthesis of peptide libraries. These peptide libraries are also important tools for the identification of new ligands. In the past years, many peptidic ligands were investigated using phage display and solid phage peptide synthesis [36-39]. For example, Fassina *et al.* have reported Protein A mimetic ligand, $(RTY)_4K_2KG$ [36] and Peptide H, a cyclic dimeric peptide of formula $(CFHH)_2KG$ with the two N-terminal cysteine residues covalently linked through a disulfide bridge [37] for IgG affinity purification. However, these ligands have not made huge inroads into the market for Protein A due to low selectivity and binding capacity. Therefore, further investigation is required, including the need to obtain more effective peptides as alternative ligands to protein A.

1.3. Applications of antibody binding peptides to antibody detection

1.3.1. Importance of antibody detection and issues of antibody detection methods

Antibodies are important molecule in diagnosis, and detection of particular antibodies is a very common form of medical diagnostics. For example, p53 antibody, HIV antibody, and antibodies of autoimmune disease are considerable candidates in diagnosis [23, 40].

Currently, enzyme-linked immunosorbent assays (ELISAs) using antibodies are widely used to quantify the concentration of target protein in blood sera, in which various proteins are present. However, heterogeneous biological immunoassays such as ELISA have been conventionally required relative long reaction time and sequential washing steps since their assays were performed in solid-phase.

On the other hand, homogeneous assays have been attracted attention because of no separation, no washing step, short assay time and ease of handling. A number of homogeneous systems have been studied in past 30 years. For example, detection schemes such as fluorescence polarization [41], fluorescence energy transfer [42] and time resolved fluorescence [43], and the fluorescent techniques is used widely in biological research. In particular, detection molecules (probes) based on energy transfer have been attracted, and a lot of probes for antibody detection, based on Forster resonance energy transfer (FRET) [44-46] and Photoinduced electron transfer (PeT) have been reported [47, 48].

1.3.2. Fluorescence-based peptide probe

Fluorophore labeled peptides have been attracted as probes for efficient and selective

detection. Single or dual fluorophore labeled peptides, whose fluorescence can change by the peptide cleavage and bond to target proteins, are widely used in various target detection assays and bioimaging assays [49-53].

Over the decade, a lot of probes for detection of a particular enzyme using dual-labeled substrate peptides have been reported. As well as enzyme detection probe, peptide probes for detection of a specific antibody have been developed [23, 44-47, 54, 55]. For example, Wei *et al.* and Plaxco *et al*. have reported peptide probes for detection of HIV antibodies [45, 55]. These peptide probes required the contrivance to undergo a conformational change as a result of the protein binding. The contrivance was not sensitive because it is due to a little changes of the distance between the fluorophores by target protein. Therefore, further improvements of the peptide probes have been required.

1.4. Aim of this thesis

In this thesis, the aim is to identify IgG binding peptides, and apply the peptides to IgG purification and homogeneous IgG detection.

In the Chapter 2, peptide ligands for purification of IgG were screened from protein sequences of IgG Fcγ receptors known as the IgG-Fc region binding proteins. High affinity octameric peptides for mouse and human IgG-Fc were obtained via three step screening process using the peptide arrays. Finally, the ability of identified peptides as ligands for IgG purification was characterized by affinity chromatography.

In the Chapter 3, homogeneous IgG detection system was developed using two peptide probes, which are fluorophore labeled IgG recognition peptide and its binding

quenching peptide. Atto655 and tryptophan were selected as fluorophore and its quencher, respectively, because tryptophan can serve as an efficient electron donor in photoinduced electron transfer (PeT) reactions with Atto655. First, in order to design the complementary quenching peptide, we identified the complementary peptide of IgG binding peptide from IgG-Fc regions, and designed the quenching peptide probes that were incorporated tryptophan residues to the N- or C-terminus using peptide array. Subsequently, IgG was detected using the designed peptide and IgG recognition peptide.

In the Chapter 4, peptide beacon was synthesized by connecting Atto655-labeled NKFRGKYK (which was identified in Chapter 2) and its complementary peptide, DIAVEWES (which was identified in Chapter 3), that conjugated tryptophan residues at C-terminus in order to form stable closed structure and induce efficient quenching, and IgG was detected using the peptide beacon.

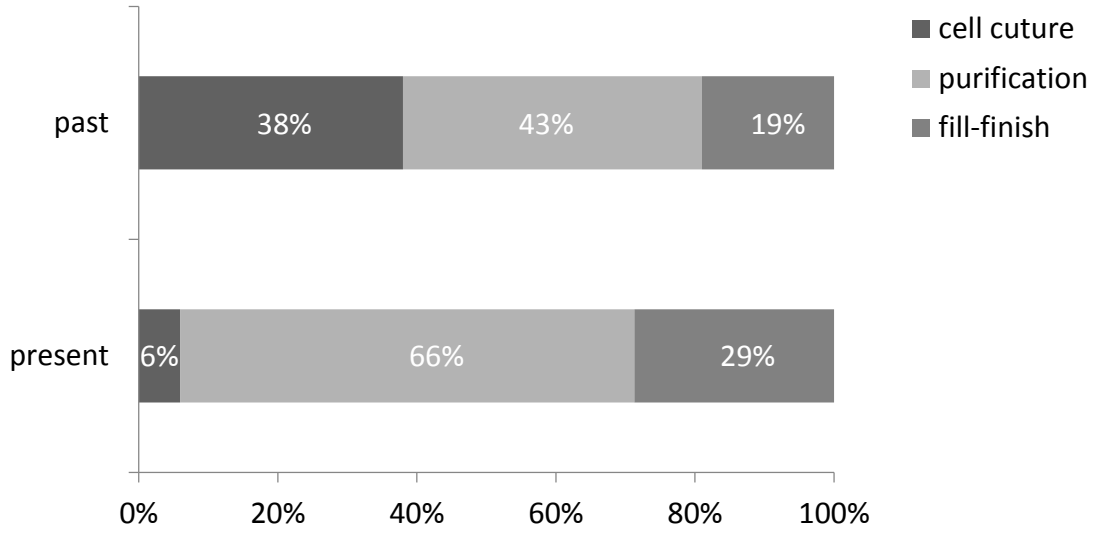

Fig. 1 The rate in each process of an antibody manufacturing cost [23]

Table 1. Comparison of Protein A with synthetic affinity ligands [2]

	Protein A	Peptidic affinity ligands	Non-peptidic affinity ligands
Preparation	Produced by recombinant bacterial systems; difficult purification	Easily synthesized	Easily synthesized
Specificity Elution	High Harsh condition (pH 2.0-3.0)	Moderate to high Mild condition	Moderate to high Mild condition
Product contamination	Leaking; high toxicity; high immunogenicity	Potentially leaking; low toxicity; low immunogenicity	Non-leaking; low/non- toxic; low/non immunogenicity
Reusability	Poor biological and chemical stability	Peptidic bonds subject to degradation	High stability
Sterilizability	Low	High	High
Scalability	limited	Easy aseptic manufacture in large quantities under GMP	Easy large-scale manufacture by chemical synthesis
Economy	High cost	Low cost	Low cost

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Chapter 2

Screening of peptide ligands that bind to the Fc region of IgG using peptide array and its application to affinity purification of antibody

2.1. Introduction

Downstream processing and purification accounts for a significant percentage (50-80%) of the total manufacturing cost for therapeutic antibodies. A major contributing factor for downstream processing costs of antibodies is the use of Protein A [1, 2]. Protein A, which specifically binds to the Fc region of IgG, is the most commonly used ligand in affinity purifications of IgG. However, there are significant problems associated with Protein A. In addition to high cost, reagents can be contaminated with bacterial endotoxin during the manufacturing of antibodies. Furthermore, there is potential risk of contamination in the final product (ligand leakage

resulting from harsh elution conditions), requiring additional purification steps, and increasing processing and validation costs. Therefore, many investigators have attempted to construct purification ligands as alternatives to Protein A [3-7]. Among the various alternative types of ligands that have been developed, small peptides have attracted great interest for affinity purification since they are more stable, less immunogenic, and less expensive compared to protein ligands. For example, Fassina *et al*. identified a protein A mimetic peptide ligand, via screening of a synthetic multimeric peptide library, and performed IgG purification from human sera [8]. Yang *et al*. identified linear hexamer peptide ligands using a hexamer peptide beads library, and showed the ability to purify human IgG from mammalian cell culture media [9]. Although many researchers have identified small ligands [10-20], these ligands have not made huge inroads into the market for Protein A. Further investigation is required, including the need to obtain more effective peptides as alternative ligands to protein A.

Peptide arrays, developed as the spot-synthesis method by Frank, are a designable peptide libraries covalently synthesized on a cellulose support and applied to various protein–peptide interaction assays [21, 22]. Our research group has identified functional peptides such as angiotensin binding peptides [23], integrin binding peptides [24, 25], amylase binding peptide [26], and TRAIL receptor binding peptides [27, 28] using peptide arrays. Peptide arrays enables high-density synthesis (approximately 300 $pmol \cdot mm^{-2}$), and thus can offer much potential for use towards IgG-Fc ligand screening.

In this study, peptide ligands for purification of IgG were screened from protein sequences of IgG Fcγ receptors known as the IgG-Fc region binding proteins [29]. High affinity octameric peptides for mouse and human IgG-Fc were obtained via three step screening process using the peptide arrays. Finally, the ability of identified peptides as

ligands for IgG purification was characterized by affinity chromatography.

2.2. Materials and methods

2.2.1 Fluorescent conjugation of antibodies

Mouse IgG (Abcam), mouse IgG-Fc (Alpha Diagnostic Intl., Inc.) and human IgG-Fc (provided by Astellas Pharmaceutical Corporation Ltd., Japan) were conjugated with Alexa Fluor® 555 succinimidyl ester (Invitrogen) according to the manufacturer's protocol. Alexa Fluor 555 succinimidyl ester was dissolved in dimethylsulfoxide (DMSO), and added to the antibody solutions. After incubation for 1 h at room temperature in the dark, unreacted Alexa Fluor succinimidyl ester dyes were removed by gel filtration using a NAP-5 column Sephadex G-25 DNA Grade (GE Healthcare). Antibody concentration was measured using the Quick Start™ Bradford protein assay method (BIO-RAD Laboratories, Inc.).

2.2.2. Peptide array synthesis

A cellulose membrane (grade 542; Whatman, Maidstone, UK) was activated using β-alanine as the N-terminal basal spacer. Fmoc-11-aminoundecanoic acid (Watanabe Chemical Inc., Hiroshima, Japan) was conjugated as an additional spacer between the candidate peptides and the cellulose. Activated Fmoc amino acids (0.25 M) were spotted to the membrane using a peptide auto-spotter (ASP222; Intavis AG, Köln, Germany) in accordance with the manufacturer's instructions with some modifications. After the first residue addition, the remaining amino groups were blocked using 2% acetic anhydride. With each elongation step, the membrane was deprotected with 20%

piperidine and subsequently washed thoroughly with N, N-dimethylformamide (DMF), followed by methanol. After the final deprotection, side-chain protecting groups were removed with a solution of m-cresol : thioanisol : ethandithiol : trifluoroacetic acid (1 : 6 : 3 : 40) for 3 h. Finally, the membrane was thoroughly washed with diethyl ether, methanol, and phosphate buffered saline (PBS, pH 7.4), respectively, and dried under sterile condition for the following assay. In the first screening, 6-mer peptide library was synthesized via stepwise synthesis of IgG-Fcγ receptor IA, B, C (Swiss-Prot No. P12314, Q92637, A6NKC4), IIA, B, C (Swiss-Prot No. P12318, P31994, P31995) and IIIA, B (Swiss-Prot No. P08637, O75015) sequences.

2.2.3. Screening of IgG binding peptides on peptide arrays

Peptide arrays were soaked in 1% bovine serum albumin (BSA) in PBS for 12 h at 4°C for blocking. After blocking, the arrays were incubated with Alexa Fluor 555 labeled mouse IgG or human IgG-Fc (a final concentration; 5 $\mu g \cdot ml^{-1}$ in PBS containing 1% BSA) for 1 h at 37°C in the dark. After washing with a Tris buffered saline containing 0.05% Tween (T-TBS; pH 7.4), the fluorescence intensities of peptide spots were scanned with a fluorescent imager (FLA-7000, Fujifilm, Tokyo, Japan) using 532 nm excitation and 585 nm emission filter pairs. The scanned images were quantified using Array Gauge ver.2.1 (Fujifilm, Tokyo, Japan).

2.2.4. Peptide affinity measurement

Triplicate peptide spots (6 mm diameter) of selected candidate peptides were synthesized on peptide arrays. Peptide arrays were soaked in 1% BSA in PBS for 12 h at

4 °C for blocking. Alexa Fluor 555 labeled human IgG-Fc was dissolved in PBS with 1% BSA so as to attain a final concentration of 0 (control), 7.67, 15.3, 30.7, 61.4, and 123 nM. After blocking, peptide arrays were incubated in the labeled human IgG-Fc at 27°C. Time lapse data was obtained by measuring the fluorescence at every time point. K_A of each peptide was obtained as follows using the formula, where F; fluorescence intensity, A_0; initial concentration of Alexa Fluor 555 labeled IgG-Fc:

$$dF/dt = k_{association} A_0 (F_{maximum} - F) - k_{dissociation} F$$

Since dF/d*t* is determined as 0 at the equilibrium state, the formula determined at the equilibrium state was follows:

$$F_{equilibrium} /A_0 = F_{maximum} /K_D - F_{equilibrium} /K_D$$

The dissociation constant (K_D) is calculated from a plot of $F_{equilibrium} /A$ versus $F_{equilibrium}$.

2.2.5. Docking simulation between IgG and peptides

Peptides were built with protein builder program and the structure of IgG with accession code 2RGS in the Protein Data Bank (PDB). The program Protonate 3D was performed to obtain their optimum hydrogen conformation at pH7.4 with the temperature of 300K, and MMFF94x force field was selected. The candidate binding pocket of IgG was identified with the program Site Finder. Molecular docking simulation was carried out to each candidate binding pocket with the program Dock.

2.2.6. Synthesis of peptide resin

The peptides were synthesized directly onto Toyopearl AF Amino 650 M resin (Tosoh, Tokyo, Japan) with a mean particle diameter of 65 μm and 1000 Å pores at a substitution density of 83 $\mu mol \cdot ml^{-1}$ using standard Fmoc coupling chemistry as

previously described [30]. The ligand density was measured by means of UV monitoring the Fmoc removal at 301 nm (ε = 7800 $cm^{-1} M^{-1}$) after deprotection. For synthesis, activated Fmoc amino acids were coupled to the amino functionality on the resin. A Kaiser test was performed at each elongation step to confirm amino acid coupling. With each elongation step, the Fmoc protecting groups were released with 20% piperidine and subsequently washed thoroughly with DMF. After the final deprotection, side-chain protecting groups were removed with a solution of m-cresol: thioanisol: ethandithiol: trifluoroacetic acid (1: 6: 3: 40) for 3 h. Finally, the resin was thoroughly washed with diethyl ether, methanol, and PBS (pH 7.4), respectively.

2.2.7. Determination of dynamic binding capacities

The volume of 100 µl resins was measured by loading the resins to the scale marks of syringe. A 100 µl volume of peptide conjugated resins were loaded into 20 mm × 5.0 mm I.D. columns (Tricorn 5/20 column, GE health care). Before applying IgG-Fc, columns were equilibrated with pH 7.0 binding buffer (20 mM phosphate buffer (PB)). Human IgG-Fc solution (1.2 $mg \cdot ml^{-1}$ in PB) was loaded onto columns at a flow rate of 0.2 $ml \cdot min^{-1}$. Dynamic binding capacity was determined at the point where human IgG-Fc concentration in the flow-through fraction reached 10% of its primary concentration.

2.2.8 Absorption and elution characteristics of IgG-Fc using the synthesized peptide resin

Each column was filled with a 200 µl volume of peptide resin. After equilibrating

the column with the binding buffer, 200 μl of human IgG-Fc solution was loaded into the column. The column was washed with 10 ml of binding buffer. A 10 ml volume of 0.1 M acetate buffer (pH 4.0), 0.1 M citrate buffer (pH 5.0), or 0.1 M PB (pH 6.0) was subsequently used for elution. Two drops (180 μl) of each fraction were collected and immediately neutralized with 20 μl of 1.0 M Tris buffer, pH 9.0. Absorbance was measured using a spectrophotometer (ND-1000, LMS, Tokyo, Japan) at 280 nm wavelength. The recovery of human IgG-Fc was calculated as a percentage of the eluted IgG-Fc relative to the total IgG-Fc loaded onto the resins.

2.2.9 Purification of IgG-Fc from cell culture medium

For purification of human IgG-Fc in the cell culture medium, 1.2 $mg \cdot ml^{-1}$ of human IgG-Fc was added to Eagle's minimal essential medium (MEM) (Invitrogen) with 10% fetal bovine serum (FBS). The peptide resin loaded column was equilibrated with 20 mM PB with 0.5 M NaCl (pH 7.0) at a flow rate of 0.2 $ml \cdot min^{-1}$. The human IgG-Fc solution (200 μl) was then loaded into the column at a flow rate of 0.2 ml/min. The column was washed with 10 ml of 20 mM PB with 0.5 M NaCl (pH 7.0). Elution was performed using 10 ml of 0.1 M acetate buffer, pH 4.0. Two drops (approximately 180 μl) of each fraction were collected and immediately neutralized with 20 μl of 1.0 M Tris buffer, pH 9.0. Absorbance was measured using a spectrophotometer at 280 nm wavelength. The purity of IgG-Fc in eluted fractions was analyzed using SDS-PAGE. Gels were stained using Quick CBB® stain reagent (Wako, Tokyo, Japan). The purity of human IgG-Fc was determined by densitometric analysis of Coomassie-stained gels using software ImageJ 1.32j (National Institutes of Health, Bethesda, MD, USA), as described by Naik [2].

2.2.10. Purification of antibody from cell culture supernatants

For purification of antibody in the CHO cell culture supernatants, cell culture supernatants containing MAb1 (1.53 $mg \cdot ml^{-1}$), which cultured in serum free CHO medium, were added to the column that packed the peptide resin (1 ml). The column was equilibrated with 20 mM PB with 0.1 M NaCl (pH 7.0) at a flow rate of 0.5 $ml \cdot min^{-1}$. The cell culture supernatants (9.8 ml) were then loaded into the column at a flow rate of 0.5 ml/min. The column was washed with 20 mM PB with 0.1 M NaCl (pH 7.0). Elution was performed using 0.2 M acetate buffer.

2.3. Results and discussion

2.3.1 Screening of mouse IgG binding peptides derived from IgG-Fcγ receptor I, II, and III

In order to explore IgG-Fc binding peptides, we constructed a hexameric peptide array containing outer membrane sequences derived from IgG-Fcγ receptors. Peptide sequences of 277 (IA), 183 (IB), 183 (IC), 181 (IIA), 181 (IIB), 175 (IIC), 192 (IIIA) and 192 (IIIB) residues were sectioned into hexamer peptide fragments with a 1-residue shift from the N-terminal, resulting in 740 spots as independent peptide sequences, designated as the FcγR-peptide array. Peptides binding to IgG were screened using the FcγR peptide array. As shown in Figure 1, several peptide sequences showed fluorescence intensities above 7.03×10^6 AU (average + 2S.D.), while the average fluorescence intensity of the whole peptide library was 1.87×10^6 AU. We identified three IgG binding regions; YRNGKAFKFFHW at Fcγ R IA, B, $C_{139\text{-}150}$, PSYRFKAN at Fcγ R $IIA_{88\text{-}95}$ and Fcγ R IIB, $C_{97\text{-}104}$, and NGKGRKYFHHN at Fcγ R IIIA, $B_{144\text{-}154}$.

Sondermann *et al*. reported that the Fc fragment of IgG was recognized by the Fc receptor via AFKFFH and RKYFHH sequences in the loops of the C-terminal domain of the Fcγ receptors [31]. Since these binding regions were included, the linear peptides synthesized by spot-synthesis method were confirmed to function in IgG binding assay. We obtained the unique peptide PSYRFKAN from Fcγ R II that contained a number of charged and aromatic amino acids such as lysine, phenylalanine and tyrosine. In computational studies of the binding complex of IgG-Fc and B domain of Protein A, Naik *et al*. reported that IgG-Fc binding sites were rich in aromatic amino acids and leucine, glutamine, isoleucine and lysine [32]. Therefore, it was shown that three regions YRNGKAFKFFHW, PSYRFKAN, NGKGRKYFHHN that contained positively charged and aromatic amino acids, were screened to have interaction with the IgG-Fc.

2.3.2 Second screening by scrambled sequence variants

Three regions were subjected to further screening including scrambled sequence variants. Three peptide sequences were sectioned into octamer peptide fragments with a 1-residue shift from the N-terminal and 10 scrambled sequence variants were designed on each octamer peptide. From the 110 peptides synthesized on the peptide array, six scrambled peptides exhibit higher affinity than the GKAFKFFH sequence which was the highest binder in the first screening (Figure 2). Fluorescent intensity of NARKFYKG (scramble peptide of YRNGKAFK) and NKFRGGYK (scramble peptide of NGKGRKYF) showed 1.40 and 1.15 fold increase in fluorescence over GKAFKFFH, respectively. Therefore, NARKFYKG and NKFRGGYK were further subjected to the next screening step.

2.3.3 Third screening by amino acid substitution assays

As a third screening, we applied alanine scanning to NARKFYKG and NKFRGGYK peptides. In both peptide sequences, each amino acid residue was sequentially substituted with an alanine residue, and the peptide array containing the substituted peptides was subsequently assayed. All variables of NARKFYKG showed decreased binding to IgG (Figure 3-a), meaning that all positions of NARKFYKG are essential for binding; further substitution was not investigated. In contrast, alanine substitution at the sixth position in NKFRGGYK resulted in increased binding to IgG (Figure 3-b). Therefore, the sixth residue of NKFRGGYK was further substituted with 19 other amino acids. The lysine substitution, NKFRGKYK, showed the strongest binding (Figure 3-c). As the result of this screening, we obtained the two peptides (NKFRGKYK and NARKFYKG) that have the high binding affinity to IgG. These peptides were further applied to the binding assay with human IgG-Fc for application to the purification process of antibody drugs in the pharmaceutical field. The binding affinity to human IgG-Fc was comparable to mouse IgG-Fc (Data not shown) and further affinity measurement was performed.

To understand the molecular interactions between the peptide and mouse IgG-Fc, the docking simulation was performed by using the software, Molecular Operating Environment, MOE (Ryoka Systems Inc., Tokyo). The binding site of these peptides was indicated as the CH2 or CH3 inside domain of IgG-Fc (PDB No. 2RGS). The peptide ligands presumed to interact at inside of IgG (Figure 4). However, since the peptide ligands immobilize on the resin for the purification, it would be difficult to reach the inside of the molecule due to the space hindrance. Therefore, in order to explore the regions of IgG-Fc binding peptides, we constructed an octameric peptide

array containing sequences derived from IgG-Fc and screened the peptides binding to NKFRGKYK using peptide array. As the result, the sequence of IgG-Fc 275-284 showed the high binding affinity for NKFRGKYK (Data not shown). The pocket of this region present at outside of IgG-Fc, and some reports show that the pocket is interaction site of IgG to ligands [33, 34]. Therefore, NKFRGKYK interacted with the region which is different to IgG-Fcγ receptor binding domain. The region contains some negative charge and hydrophobic amino acid. Since NKFRGKYK contains some positive and aromatic amino acid, it is suggested that the region and NKFRGKYK are bound with the electrostatic interaction and the hydrophobic interaction.

2.3.4 Affinity measurement of NKFRGKYK and NARKFYKG for human IgG-Fc using peptide arrays

The association rate constant (K_A) of NKFRGKYK and NARKFYKG peptides was determined using peptide arrays. Each peptide was spot-synthesized on the surface of a peptide array and incubated with Alexa Fluor 555 labeled human IgG-Fc at a series of concentrations (0-123 nM; Figure 5). An increase in fluorescent intensity was observed over time, reaching equilibrium after 90 minutes at concentrations below 61.4 nM. Based on these results, the association constant values for NKFRGKYK and NARKFYKG were calculated as 8.9×10^6 and 6.5×10^6 M^{-1}, respectively. These association constants were within the normal range (10^4 - 10^8 M^{-1}) for affinity chromatography resins [17]. Therefore, it was shown that these peptides have sufficient affinity to IgG-Fc.

2.3.5 Determination of dynamic binding capacity using peptide conjugated resins

The utility of NKFRGKYK and NARKFYKG as the purification ligand in affinity chromatography was investigated by synthesizing these peptides on Toyopearl AF Amino 650M resin. The dynamic binding capacities of human IgG-Fc to NKFRGKYK and NARKFYKG resins were determined by breakthrough experiments. A 1 ml volume of human IgG-Fc solution was loaded onto 100 μl of each peptide resins at a flow rate of 0.2 $ml \cdot min^{-1}$. The breakthrough curves are shown in Figure 6. The dynamic binding capacities of NKFRGKYK and NARKFYKG for human IgG-Fc at the 10% breakthrough point were estimated to be 4.9 and 5.0 $mg \cdot ml^{-1}$ of resin, respectively. Naik *et al*. reported that the dynamic binding capacities of three peptide ligands for monoclonal antibodies (humanized IgG4), were 17 to 19 $mg \cdot ml^{-1}$ of resin [2]. The dynamic binding capacities of human IgG would be 3 fold higher than that of human IgG-Fc since the molecular weight of human IgG-Fc (approximately 50 kDa) is one-third of human IgG (approximately 150 kDa). Therefore, we concluded that the dynamic binding capacities of our two peptides were comparable to the previously reported peptide ligand [2] and Protein A. Therefore, these peptides are useful as purification ligands for IgG.

2.3.6. Purification of human IgG-Fc using peptide ligands

The effect of pH on the elution of human IgG-Fc was investigated using elution buffers at pH 4.0, 5.0 and 6.0. Figure 7 shows the chromatograms of IgG-Fc elution using the NKFRGKYK and NARKFYKG resins. The recovery rate of IgG-Fc increased alongside a decrease in pH of the elution buffer, and it was 81.6% and 88.7% using NKFRGKYK and NARKFYKG resins, respectively (Table 1). Our results show that over 80% of the applied IgG-Fc was eluted at pH 4.0. Since elution characteristics

differed largely depending on pH, it was assumed that these peptides mainly bound to the IgG-Fc via electrostatic interactions.

Purification of IgG-Fc from cell culture medium was investigated using the peptide resins. Human IgG-Fc was added to the MEM containing 10% FBS at a concentration of 0.6 mg/ml, and the mixture was directly loaded into 0.2 ml chromatographic columns packed with each of the peptide conjugated resins. The columns were equilibrated with 20 mM PB with 0.5 M NaCl. After loading 200 μl of the cell culture media containing IgG-Fc, the column was washed with PB containing 0.5 M NaCl to remove nonspecific binding. Then, human IgG-Fc was eluted using 0.1 M acetate buffer at pH 4.0. Fractionation was performed every 0.2 ml and the purity of collected fractions was analyzed via SDS-PAGE. The purity of the human IgG-Fc in the collected fraction was 83% and 68% using the NKFRGKYK and NARKFYKG conjugated resins, respectively (Figure 8). From SDS-PAGE analysis, most of the contaminated protein was found to be BSA. This nonspecific interaction of BSA to the peptide resin was also reported by Naik *et al.* [2], and the use of 1.0 M NaCl or sodium caprylate for equilibration and separation was effective for preventing nonspecific BSA binding. Also, Liu *et al*. has succeeded to improve the purity of IgG from MEM by means of peptide density control [35]. Therefore, by optimization of the chromatographic procedure for NKFRGKYK and NARKFYKG conjugated resin will further increase the purification ratio.

We optimized washing condition of the peptide conjugated resin with 20 mM PB containing 0-1.0 M NaCl. However, the purity was not improved above 0.5 M NaCl (data not shown). Also, we synthesized the peptide resin with different density (40, 15, and 8.8 μmol/ml-resin). However, increase in purification efficiency was not observed using our peptides. Further optimization of washing buffer and elution buffer is

necessary to eliminate BSA. Moreover, we performed the purification of an antibody in cell culture supernatants as preliminary experiment. For purification of antibody in the cell culture supernatants, Mab in cell culture supernatants were loaded into 1.0 ml chromatographic columns packed with each of the peptide conjugated resins. After loading 9.8ml of the cell culture media containing the Mab, the column was washed with PB containing 0.1 M NaCl to remove nonspecific binding. Then, the Mab was eluted using 0.2 M acetate buffer, resulting in a single peak (Figure 9a, b). Fractionation and SDS-PAGE of the collected fractions were performed (Figure 9c). Amount of DNA and host cell protein (HCP) in collected fractions were determined by absorbance and Bradford assay. From the analysis, the yield of the MAb in the collected fraction was 69.2% and 80.1% using the NKFRGKYK and NARKFYKG conjugated resins, respectively. The HCP reductions obtained by these peptides resin were in the range of 1.3LRV (NKFRGKYK) and 1.5LRV (NARKFYKG) respectively (Table 2). These peptides resin were found to be very efficient in clearing the DNA.

One-step purification of IgG from a mammalian cell culture using the synthetic peptide ligands is a difficult challenge. However, synthetic peptide ligands are more stable and less immunogenic compared to protein ligands, and the screened NKFRGKYK and NARKFYKG peptide via the spot synthesis method could be used as the peptide ligand for the purification of IgG. Further research is necessary to specify chromatographic condition and design of new peptide ligands, which shows low nonspecific binding of both BSA and host cell proteins, and shows high IgG-Fc binding. Similarly to the binding assay with IgG, interaction of both BSA and host cell proteins and candidate peptides should be investigated via screening steps using peptide arrays. Given that synthetic peptide ligands eliminate the anxiety of biological contaminants

such as viruses, pyrogens, DNA fragments and other biomolecules, further screening and conformational designs are necessary for the practical realization of IgG purification.

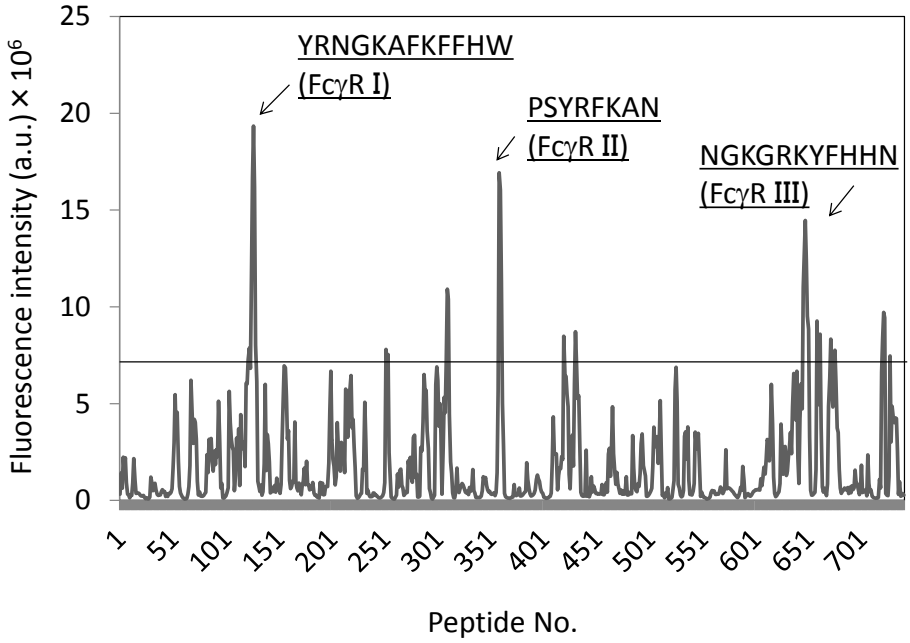


Fig. 1 Identification of mouse IgG binding sites in IgG-Fcγ receptors using a 6-*mer* peptide library. YRNGKAFKFFHW (Peptide No. 123-129) derived from Fcγ R I, PSYRFKAN (Peptide No. 359-361) derived from Fcγ R II and NGKGRKYFHHN (Peptide No. 646-651) derived from Fcγ R III show high binding affinity to mouse IgG.

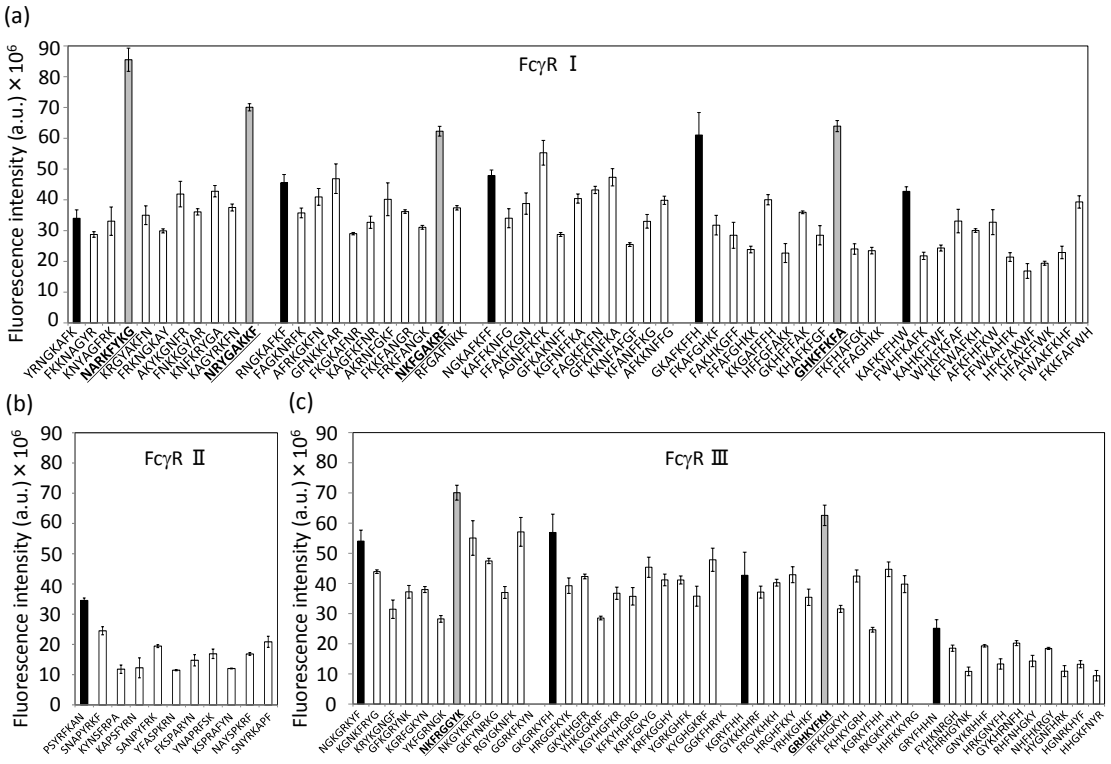
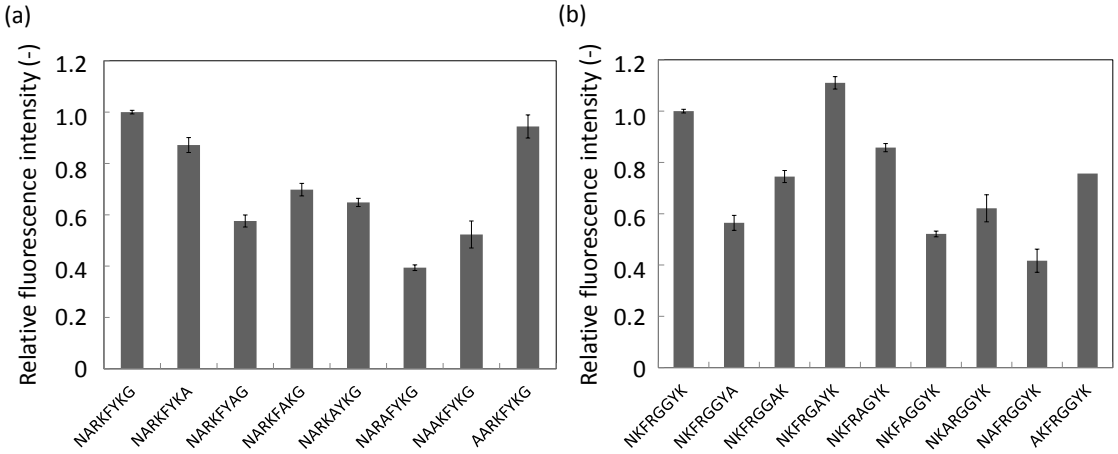


Fig. 2　Binding affinity of scrambled sequence variants from mouse IgG binding peptides selected in the first screening. Black bars show the peptides selected during first screening. Gray bars show peptides with a higher affinity than GKAFKFFH, which was the highest binder in the first screening.



(c)

NKFRGXYK	Substituted amino acid									
	R	K	H	P	I	A	L	G	V	W
	1.13	1.44	0.94	0.87	1.08	1.01	1.07	1.00	1.14	0.55
	M	S	T	Y	Q	F	N	C	E	D
	1.07	0.95	0.99	1.00	0.91	1.16	0.84	1.09	0.52	0.52

Fig. 3 Alanine scanning of the NARKFYKG (a) and NKFRGGYK (b) peptides. Fluorescent intensity of NARKFYKG and NKFRGGYK was calculated as 1.0. Amino acid substitution analysis at the sixth position of NKFRGGYK using a peptide array (c). The light gray matrix shows a 1.0 fold increase over fluorescent intensity of NKFRGGYK, while the dark gray matrix showing a lysine substitution exhibited the highest binding.

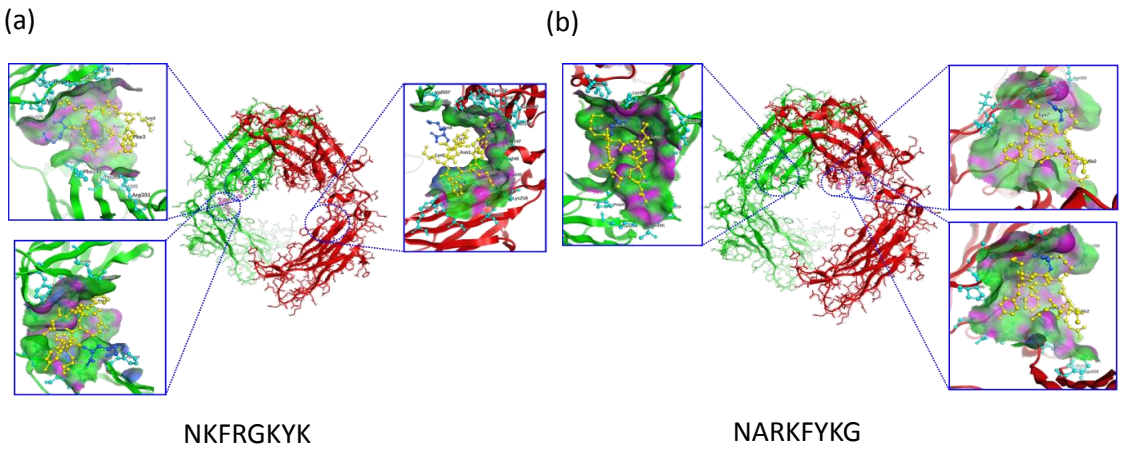


Fig.4 Docking simulation between NKFRGKYK (a) or NARKFYKG (b) and IgG-Fc using the Molecular Operating Environment (MOE) software.

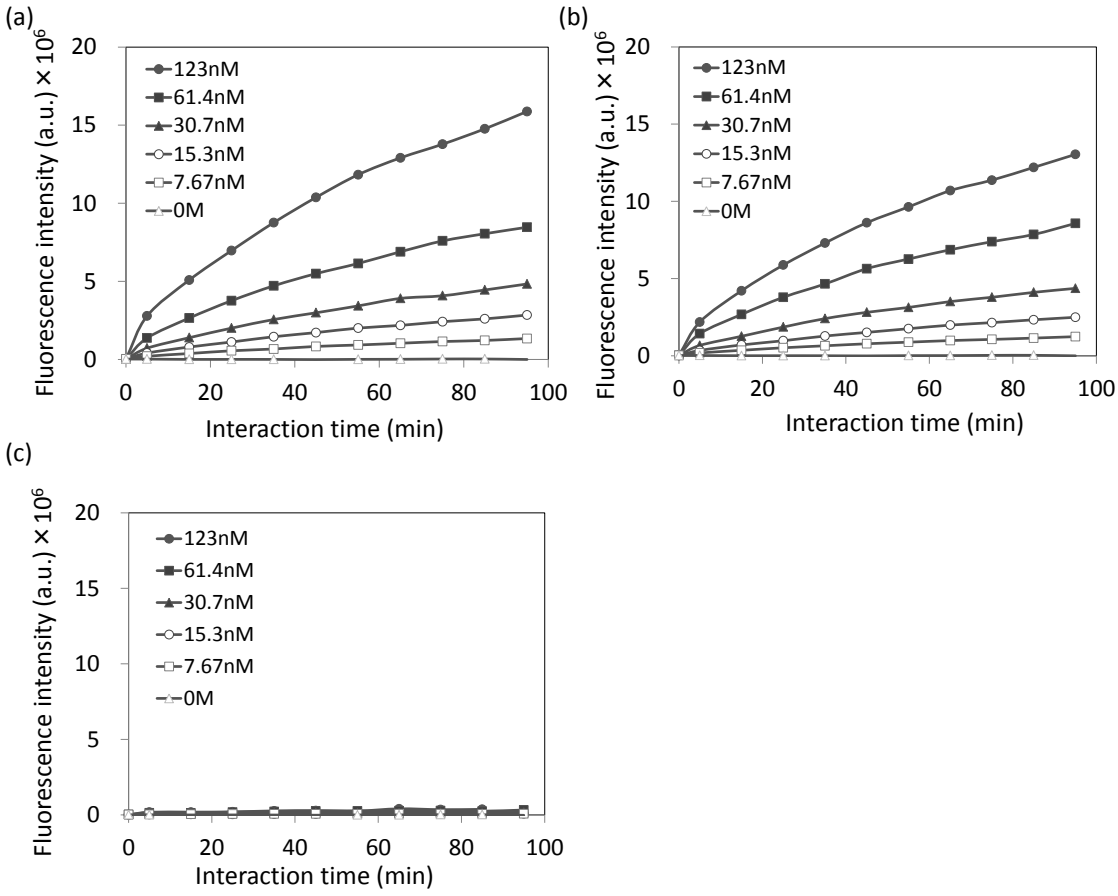


Fig. 5 Association curve of the NKFRGKYK (a), NARKFYKG (b), and AAAAAAAA (c) peptides. A time series recording fluorescent intensity was measured using the peptide array with Alexa Flour 555-labeled human IgG-Fc solution at different concentrations (μM): 0 (open triangles), 7.67 (open squares), 15.3 (open circles), 30.7 (closed triangles), 61.4 (closed squares), and 123 (closed circles).

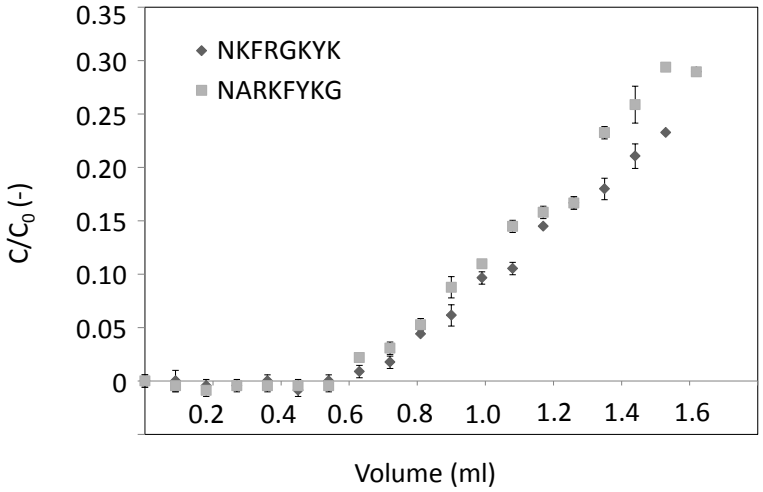


Fig. 6 Breakthrough curves of the NKFRGKYK and NARKFYKG peptide conjugated resins for human IgG-Fc.

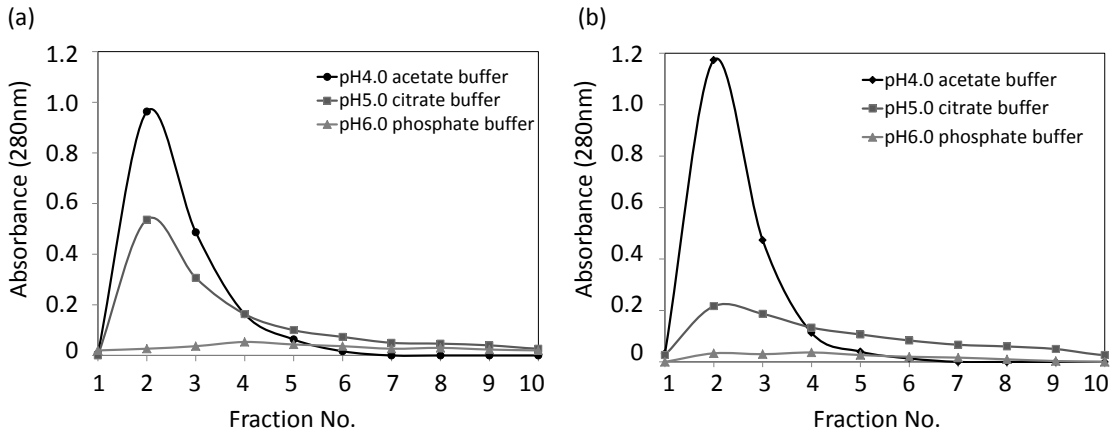


Fig. 7 Chromatograms of human IgG-Fc elutions using the NKFRGKYK (a) and NARKFYKG (b) conjugated resins at different pH; pH4.0 acetate buffer (black circles), pH5.0 citrate buffer (dark gray squares), pH6.0 phosphate buffer (light gray triangles).

Table 1 Recovery of human IgG-Fc using NKFRGKYK– and NARKFYKG–conjugated resins at different pH buffer.

Peptide	pH	Elution buffer	Recovery(%)
NKFRGKYK	6.0	0.1M phosphate	16.9
	5.0	0.1M citrate	73.7
	4.0	0.1M acetate	81.6
NARKFYKG	6.0	0.1M phosphate	9.4
	5.0	0.1M citrate	59.0
	4.0	0.1M acetate	88.7

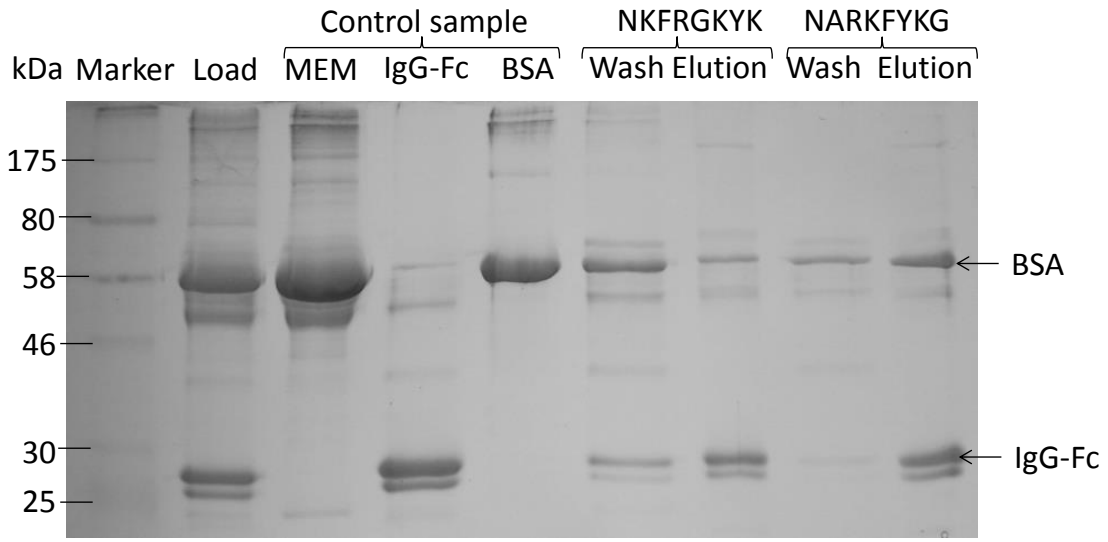


Fig. 8 SDS-PAGE analysis of the peptide-ligand affinity-purified human IgG-Fc from cell culture medium. The gel was run under reducing conditions. The purity of IgG-Fc eluted using NKFRGKYK and NARKFYKG resins at pH 4.0, was 83.3% and 68.3%, respectively. The purity was calculated by densitometry analysis using ImageJ.1.32j.

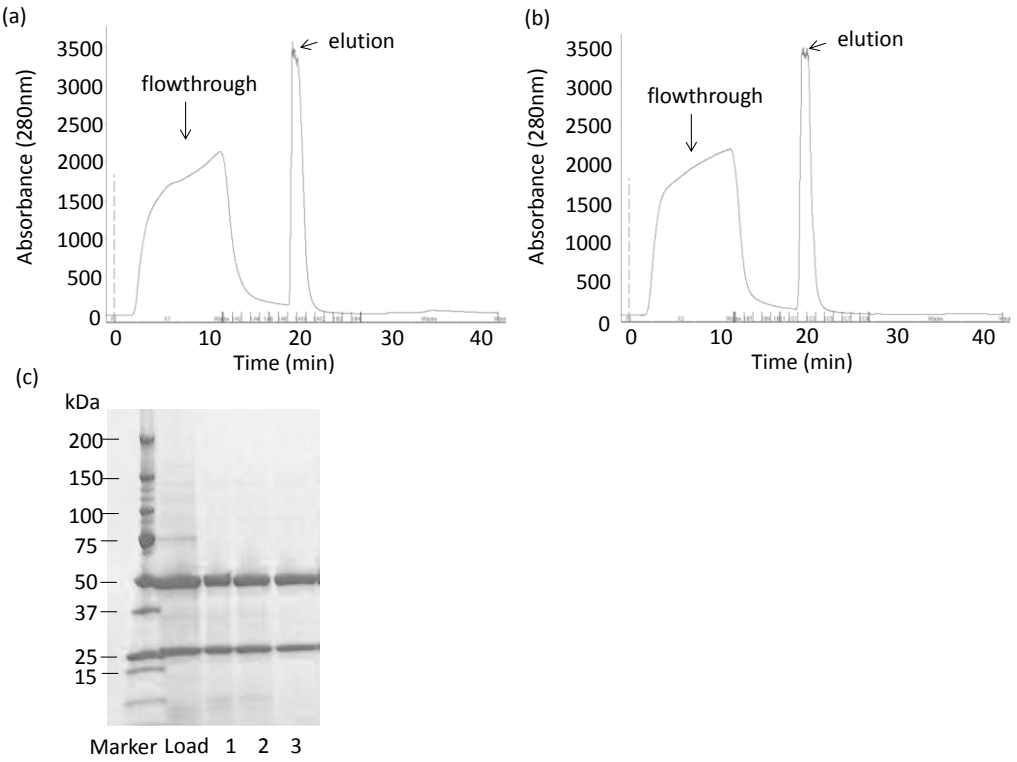


Fig.9 Chromatograms of purification of MAb from cell culture supernatants (1.53mg/ml) using (a) NKFRGKYK and (b) NARKFYKG resin. (c) SDS-PAGE (reducing condition) of flow through and elution fractions. Lane 1: NKFRGKYK, Lane 2: NARKFYKG, Lane 3: Protein A.

Table 2 DNA content and host cell protein of the chromatographic fractions of purification of MAb1 using peptide resin.

	DNA (pg/mg MAb1)	HCP (ng/mg MAb1)
Load	3143466	33516
Elution (NKFRGKYK)	185	1554
Elution (NARKFYKG)	416	996

2.4. Summary

In this study, screening of the IgG-Fc binding peptides was investigated using a spot peptide array and its usefulness as the IgG purification ligand was evaluated. As the result of 3 steps screening, the two peptides, NKFRGKYK and NARKFYKG that have high binding affinity to IgG were obtained. The association constant values of NKFRGKYK and NARKFYKG for human IgG-Fc were 8.9×10^6 and 6.5×10^6 M^{-1}, respectively. The dynamic binding capacities of NKFRGKYK, NARKFYKG for human IgG-Fc were estimated to be 4.9 mg/ml resin, 5.0 mg/ml resin, respectively.

Purification experiment to test the ability of the peptide resins to isolate human IgG-Fc from MEM containing 10% FBS was also carried out. The chromatographic isolation of human IgG-Fc in purification step achieved purity of around 80% at NKFRGKYK or 70% at NARKFYKG, respectively. These peptide ligands were used for the purification of an antibody from cell culture supernatants. The yields obtained for the antibody were found to be 69% and 80% respectively. The residual DNA and HCP reduction obtained by these peptides resin were in the range of 4.2 and 1.3LRV (NKFRGKYK), 3.9 and 1.5LRV (NARKFYKG) respectively. Therefore, it was indicated that the screened NKFRGKYK and NARKFYKG peptide would be useful as affinity purification ligands for IgG.

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Chapter 3

Design of quenching peptide probes incorporating tryptophan for rapid antibody detection

3.1. Introduction

The development of simple, rapid, sensitive, and inexpensive bioassays is of great importance for medical diagnostics and pharmaceutical development. Common protein detection methods have mainly been dominated by immunological assay methods [1-3]. These methods have high specificity and sensitivity. However, these heterogeneous techniques are time consuming due to the required washing steps, long reaction times, and expensive reagents. Therefore, a homogeneous assay method for easy and rapid measurement of proteins would represent a powerful tool [4].

Large numbers of functional short peptides that bind to a variety of protein targets have been discovered by high throughput screening techniques [5-7]. These specific peptides could become molecular recognition probes for protein sensing. Despite the

potential advantages, there are few approaches for target protein detection since most of peptide-protein bindings do not readily produce measurable signals.

Recently, fluorescence-based peptide probes have attracted attention in biomedical fields as a homogeneous detection tool in a simple way. The fluorescence signal of the peptide probes in which various fluorophores are conjugated to N- and C-terminus is obtained depending on the distance between the fluorophores, such as FRET and quenching effects. Recently, some peptide-based optical sensing approaches were investigated using energy transfer or electron transfer between the fluorophore and quencher modified the peptide [8-14]. However, few peptide probes for non-enzyme proteins have been reported. This is due to the fact that a little changes of the distance between the fluorophores by target protein binding. Therefore, these detection strategies have a limited scope for adaptation. On the other hand, approaches that detect a target protein using fluorescent-labeled peptides and another quenching material have attracted as universal detection method. For example, Lu *et al*. and Wang *et al*. have reported that graphene oxide can be used as a quencher to selectively suppress the fluorescence of unbound peptide probes, enabling fast, sensitive, and selective detection of proteins [15, 16]. Zhu *et al.* have reported that a carbon nanotube can be used as a quenching platform for fluorescent peptide probes [17]. However, protein detection approaches based on peptide-peptide interactions have not been reported such as complementary molecules in DNA detection.

Peptide arrays, developed as the SPOT method by Frank, are a designable peptide library that is covalently synthesized on a cellulose support, and have been applied to various interaction assays. Our research group has designed various peptides interacting to protein, cell receptors, peptides and nanoparticle using peptide arrays [18-22]. The

IgG-Fc high binding peptide (NKFRGKYK) was also designed from IgG Fcγ receptors, and used as the purification ligand of IgG from cell culture medium [23].

In this study, homogeneous protein detection system was developed using two peptide probes, fluorophore labeled molecular recognition peptide and its binding quenching peptide. Atto655 and tryptophan were selected as fluorophore and its quencher, respectively, since tryptophan can serve as an efficient electron donor in photoinduced electron transfer (PeT) reactions with Atto655 [24, 25] and PeT-based analysis applied for immunological detection of target molecules as elegant sensor [26]. IgG was used as the model protein and the Atto655-labeled NKFRGKYK was selected as the probe for recognition of Fc region of IgG. First, in order to design the complementary quenching peptide, we identified the NKFRGKYK binding peptide from IgG-Fc regions, and designed the quenching peptide probes that were incorporated tryptophan residues to the N- or C-terminus using peptide array. Subsequently, IgG was detected using the designed peptide and Atto655-labeled NKFRGKYK.

3.2. Materials and methods

3.2.1. Peptide array synthesis

A cellulose membrane (grade 542; Whatman, Maidstone, UK) was activated using β-alanine as the N-terminal basal spacer. Fmoc-11-aminoundecanoic acid (Watanabe Chemical Inc., Hiroshima, Japan) was conjugated as an additional spacer between the candidate peptides and the cellulose. Activated Fmoc amino acids (0.25 M) were spotted to the membrane using a peptide auto-spotter (ASP222; Intavis AG, Köln, Germany) in accordance with the manufacturer's instructions with some modifications.

A carboxyl group of activated Fmoc amino acid was combined with 11-aminoundecanoic acid on the cellulose membrane. After the first residue addition, the remaining amino groups were blocked using 2% acetic anhydride. With each elongation step, the membrane was deprotected with 20% piperidine and subsequently washed thoroughly with N, N-dimethylformamide (DMF), followed by methanol. After the final deprotection, side-chain protecting groups were removed with a solution of m-cresol : thioanisol : ethandithiol : trifluoroacetic acid (1 : 6 : 3 : 40) for 3 h. Finally, the membrane was thoroughly washed with diethyl ether, methanol, and phosphate buffered saline (PBS, pH 7.4), respectively, and dried under sterile condition for the following assay. In the first screening, an 8-mer peptide library was synthesized via stepwise synthesis of IgG-Fc sequences (Swiss-Prot No. P01857).

3.2.2. Screening of NKFRGKYK binding peptides on peptide arrays

Peptide arrays were soaked in 1% bovine serum albumin (BSA) in PBS for 12 h at 4°C for blocking. After blocking, the arrays were incubated with Atto655-labeled NKFRGKYK peptides (a final concentration; 1 μM in PBS) for 1 h at 37°C in the dark. After washing with PBS, the fluorescence intensities of peptide spots were scanned with a fluorescent imager (FLA-9500, GE Healthcare UK Ltd., England) using 635 nm excitation and 665 nm emission filter pairs. The scanned images were quantified using Image Quant TL (GE Healthcare UK Ltd., England).

3.2.3. Design of quenching peptides on peptide arrays

NKFRGKYK binding peptides were modified tryptophan residues to design

quenching peptides. Designed peptides were synthesized on a peptide array. Each peptide spot was punched out as a peptide disk and inserted into a 96-well plate. Peptide arrays were soaked in PBS containing 1% BSA for 1 h at 37°C for blocking. After blocking, the arrays were incubated with Atto655-labeled NKFRGKYK peptides (1 μM in PBS) for 1 h at 37°C in the dark. After incubation, the fluorescence intensities of peptide spots were scanned with a fluorescent spectral reader (POWER SCAN 4, DS Pharma Biomedical Co., Ltd., Osaka, Japan) at 650 nm excitation. Fluorescence intensity of 684nm in fluorescence spectra of NKFRGKYK binding peptides was set to 1.0, and the quenching values of designed peptides were calculated.

3.2.4. Quenching effect of quenching peptides in solution

Quenching peptides were purchased from Sigma-Aldrich Japan. In quenching experiments, Atto655-labeled NKFRGKYK peptides (final concentration 0.5 μM) and a serial dilution of quenching peptides (final concentration 0 to 500 μM) were mixed in a cuvette. The fluorescence emission spectrum of the Atto655-labeled peptide was recorded on a spectrofluorophotometer (RF-5300PC, Shimadzu Corporation, Kyoto, Japan) at 650 nm excitation.

3.2.5. IgG detection by using quenching peptides

Atto655-labeled NKFRGKYK peptides and a serial dilution of human IgG were pre-incubated for 10 min into 384-well plate before the addition of quenching peptides. After incubation, quenching peptides (final concentration 300 μM, DMSO 8%) were added to the mixing solution of Atto655-labeled NKFRGKYK peptides (final concentration 1.0 μM) and human IgG (final concentration 0 to 750 nM). The

fluorescence emission spectrum of the Atto655-labeled peptide was recorded on the fluorescent spectral reader at 650 nm excitation after 30 s incubation.

3.3. Results and discussion

3.3.1. Screening of NKFRGKYK binding peptides derived from IgG-Fc

In order to explore NKFRGKYK binding peptides, we synthesized a octameric peptide array containing sequences derived from IgG-Fc. Peptide sequences of 220 residues were sectioned into octamer peptide fragments with a 1-residue shift from the N-terminus, resulting in 213 spots as independent peptide sequences, designed as the IgG-Fc peptide array. Peptides binding to NKFRGKYK were screened using the IgG-Fc peptide array. As shown in Figure 1, several peptide sequences showed fluorescence intensities above 1.4×10^6 AU (average + 2 S.D.) while the average fluorescence intensity of the whole peptide library was 2.2×10^5 AU (Fig. 1). We identified two NKFRGKYK binding regions: FNWYVDGVEV (No. 49-51) and SDIAVEWESNGQP (No. 149-153) (Table 1). These peptides contained some negative charges and hydrophobic amino acids. Since NKFRGKYK contains some positive charges and aromatic amino acids, it suggests that these peptides are bound by electrostatic and hydrophobic interactions. Since NWYVDGVE and DIAVEWES showed high affinity for NKFRGKYK during screening, these peptides were further subjected to the next screening step.

3.3.2. Design of quenching peptides incorporating tryptophan at the N- and C-terminus using the spot peptide array

In order to design quenching peptides, the peptides incorporating 1-3 residues of tryptophan at the N-terminus or C-terminus of NWYVDGVE and DIAVEWES were synthesized on the spot peptide array. After embedding the spot synthesized peptide disk in a 96-well plate, Atto655-labeled NKFRGKYK peptide solution was added into each well and the fluorescence spectrum was measured. Little quenching was observed in poly-A (ocatamer) peptide incorporating 3 residues of tryptophan (which is a negative control peptide). In contrast, efficient quenching was observed in the designed quenching peptides (Fig. 2). Comparing WG-peptides and the peptide-GW in Figure 2, it suggests that NWYVGVE binds to NKFRGKYK by reverse parallel alignment, since quenching of Atto655-labeled NKFRGKYK was observed in NWYVGVE-GW and not observed in WG-NWYVGVE (Fig. 2 a, -b). Therefore, effective quenching was not observed in the case where tryptophan residues were added to the N-terminus. On the other hand, it suggests that DIAVEWES binds to NKFRGKYK by ordered parallel alignment since quenching of Atto655-labeled NKFRGKYK was observed in WG-DIAVEWES and not observed in DIAVEWES-GW. However, in the case where a quenching group was large, such as in WWW, quenching of Atto655-labeled NKFRGKYK was observed regardless of the C-terminus and N-terminus addition (Fig. 2 c, -d). This may be due to the fact that these peptides are folded after binding to each other. Since WWG-DIAVEWES and WWWG-DIAVEWES showed effective quenching of fluorescence, these peptides were further subjected to the next assay.

3.3.3. Quenching effect of WWG-DIAVEWES and WWWG-DIAVEWES in solution

In order to investigate the effect of quenching peptides, soluble peptides of

WWG-DIAVEWES and WWWG-DIAVEWES were prepared. These peptides were mixed in a solution containing Atto655-labeled NKFRGKYK at various concentrations for 10 min, and the fluorescence spectrum was measured using a spectrofluorophotometer. In both cases of adding WWG-DIAVEWES and WWWG-DIAVEWES, a dose-dependent decrease in the fluorescence was observed (Fig. 3 a,-b). Figure 3 (c) shows the relationship of the fluorescence intensity at 684 nm, which is the fluorescence maximum of Atto655 dye, and the quenching peptide concentration. The quenching activity of WWWG-DIAVEWES was shown to be higher than WWG-DIAVEWES, and this further indicated that incorporation of three tryptophan residues was effective for the quenching of Atto655-labeled NKFRGKYK. Fluorescence intensity was sufficiently decreased at a peptide concentration of 300 μM in the case where WWWG-DIAVEWES was added. Little quenching was observed when WWWG was added as a control, even when WWWG was added at 500 μM. Therefore, it was shown that these quenching peptides interacted with Atto655-labeled NKFRGKYK and quenching of Atto655 was observed for tryptophan in close proximity. Dissociation constants (Kd) of 8.3×10^{-5} M (WWGD-IAVEWES) and 1.2×10^{-5} M (WWWG-DIAVEWES) were obtained from Fig. 3-(c).

In order to investigate the time required for quenching of fluorescence, the change in fluorescence intensity after the addition of WWWG-DIAVEWES was measured. Sufficient quenching of fluorescence was observed at 30 s after the addition of WWWG-DIAVEWES, and the fluorescence intensity was constant after 30 s (Fig. 4). Therefore, these peptides interacted rapidly with each other and this reaction could be measured within 30 s.

3.3.4. IgG detection

IgG was detected using Atto655 labeled NKFRGKYK and WWWG-DIAVEWES. Atto655-labeled NKFRGKYK and IgG was preincubated for 10 min at 384-well plate. Subsequently, WWWG-DIAVEWES (final concentration 300 μM, DMSO 8%) were added to the mixture solution of Atto655-labeled NKFRGKYK peptides (final concentration 1.0 μM) and human IgG (final concentration 0 to 750 nM). The fluorescence emission spectrum of the Atto655-labeled peptide was recorded on the fluorescent spectral reader at 650 nm excitation after 30 s incubation. An increase in the fluorescence spectrum was observed following the concentration of IgG (Figure 5a). This is due to the inhibition of WWWG-DIAVEWES and Atto655-labeled NKFRGKYK binding by addition of IgG. In contrast, no increase in the fluorescence spectrum was observed when IgG was added after preincubation of the two peptides (data not shown). Figure 5-(b) shows the relationship between the fluorescence intensity at 684 nm and IgG or BSA concentration. The dose-dependent increase of the fluorescence was observed for IgG concentrations above 10 nM. This detection sensitivity is almost the same as the dissociation constant of NKFRGKYK for IgG-Fc (1.1×10^{-7} M). In contrast, little increase in the fluorescence following the concentration of BSA was observed. The increase in the fluorescence was saturated at 250 nM IgG. It was suggested that four molecules of Atto655-labeled NKFRGKYK would be bound to one molecule of IgG since Atto655-labeled NKFRGKYK was added to IgG solution at 1μM in the assay. Since two NKFRGKYK binding sites from IgG-Fc region were identified in first screening (Fig. 1) and IgG is composed two Fc regions as one molecule by disulfide bonds, it was expected result. As a result, it was shown that IgG could be detected specifically using the two peptide. In this study, the detection limit of

IgG was 10 nM since the dissociation constant of NKFRGKYK and IgG-Fc was not so high (1.1×10^{-7} M). Further improvement of sensitivity is expected by designing high affinity peptide for IgG-Fc to apply this technique in diagnostics.

Recently, homogeneous assay have attracted attention as an easy and rapid measurement of target proteins. In this study, the quenching peptide probe, which is the key molecule for detection IgG, was designed, and IgG could be detected specifically using Atto655-labeled NKFRGKYK and WWWG-DIAVEWES. This homogeneous assay only requires mixing and fluorescence measurement, and does not required washing steps and long incubation times. Furthermore, this approach would be applicable to variety of target proteins just by design of fluorescence and quenching peptide probes. Therefore, this detection approach was useful for protein detection due to its short reaction time and simplicity. However, since dissociation constant between designed quenching peptide, WWWG-DIAVEWES, and Atto655-labeled NKFRGKYK was not so high (1.2×10^{-5} M), high concentration of quenching peptide was needed to quench Atto655-labeled NKFRGKYK in this IgG detection assay. In order to decrease the amount of quenching peptide, further design of quenching peptides would be necessary such as using peptide variants of DIAVEWES.

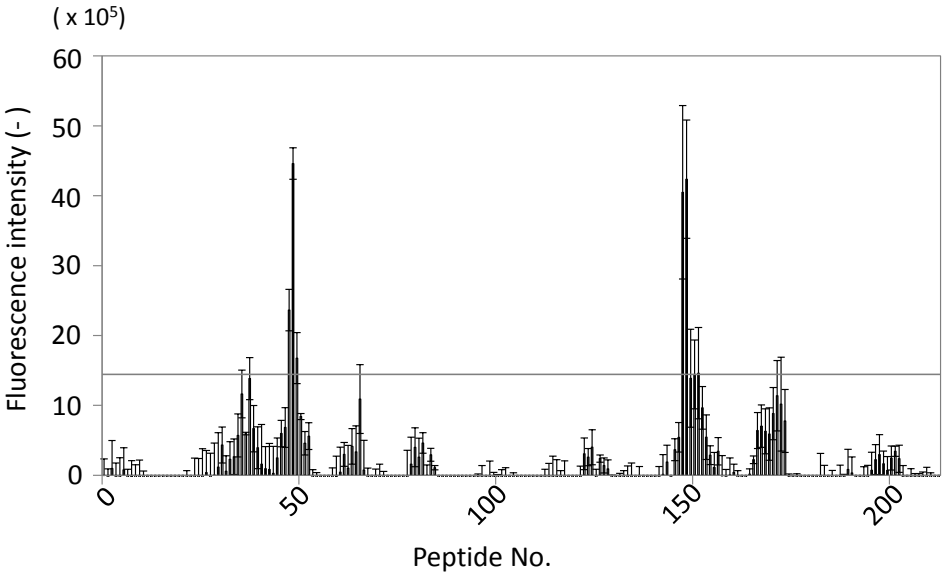

Fig. 1 Screening of NKFRGKYK binding peptides using the IgG-Fc peptide array. The average fluorescence intensity of the whole peptide library was 2.2 x 10^5. The black line indicates an intensity of 1.4 x 10^6 (= average + 2 standard deviation).

Table 1 NKFRGKYK binding peptides and their properties.

No.	Sequence	Fluorescence intensity (-)	pI	GRAVY
49	FNWYVDGV	23.6 ± 3.0	3.8	0.20
50	NWYVDGVE	44.6 ± 2.2	3.7	-0.59
51	WYVDGVEV	16.8 ± 3.7	3.7	0.38
149	SDIAVEWE	40.5 ± 12.4	3.6	-0.21
150	DIAVEWES	42.4 ± 8.5	3.6	-0.21
151	IAVEWESN	13.9 ± 7.0	3.8	-0.21
152	AVEWESNG	14.4 ± 4.9	3.8	-0.83
153	VEWESNGQ	14.6 ± 6.5	3.8	-1.49

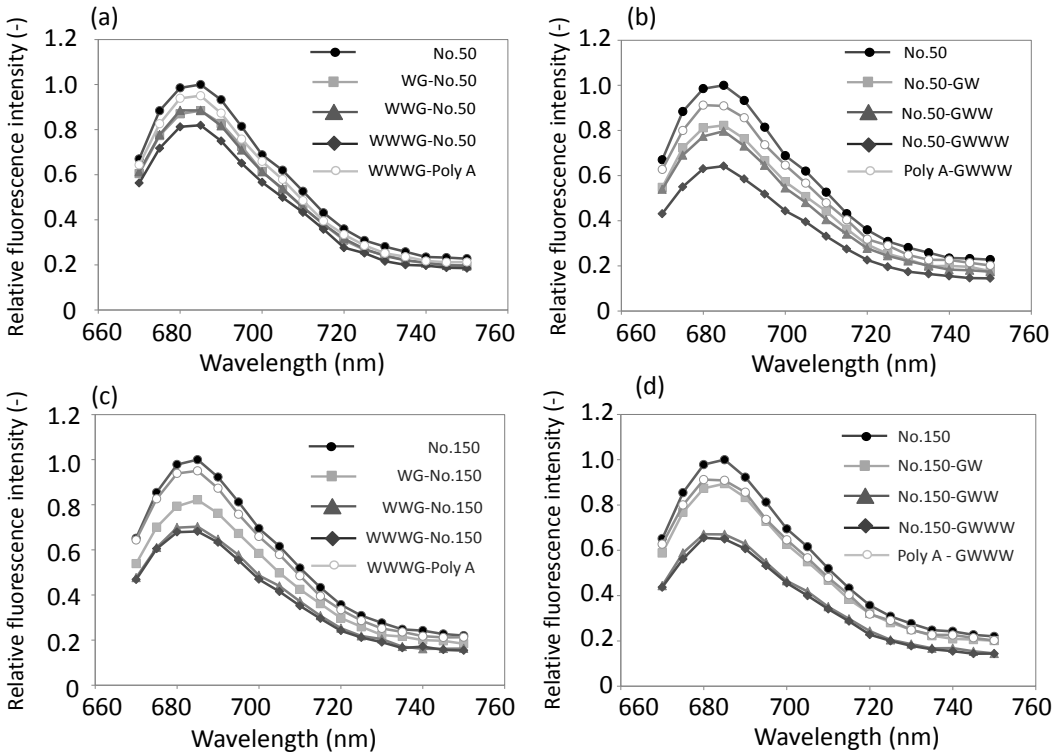

Fig. 2 Quenching activity of Atto655-labeled NKFRGKYK using NWYVDGVE (No. 50) or DIAVEWES (No. 150) incorporating tryptophan on the membrane disk. (a) The peptide that incorporated poly-tryptophan at the N-terminus of NWYVDGVE. (b) The peptide that incorporated poly-tryptophan at the C-terminus of NWYVDGVE. (c) The peptide that incorporated poly-tryptophan at the N-terminus of DIAVEWES. (d) The peptide that incorporated poly-tryptophan at the C-terminus of DIAVEWES.

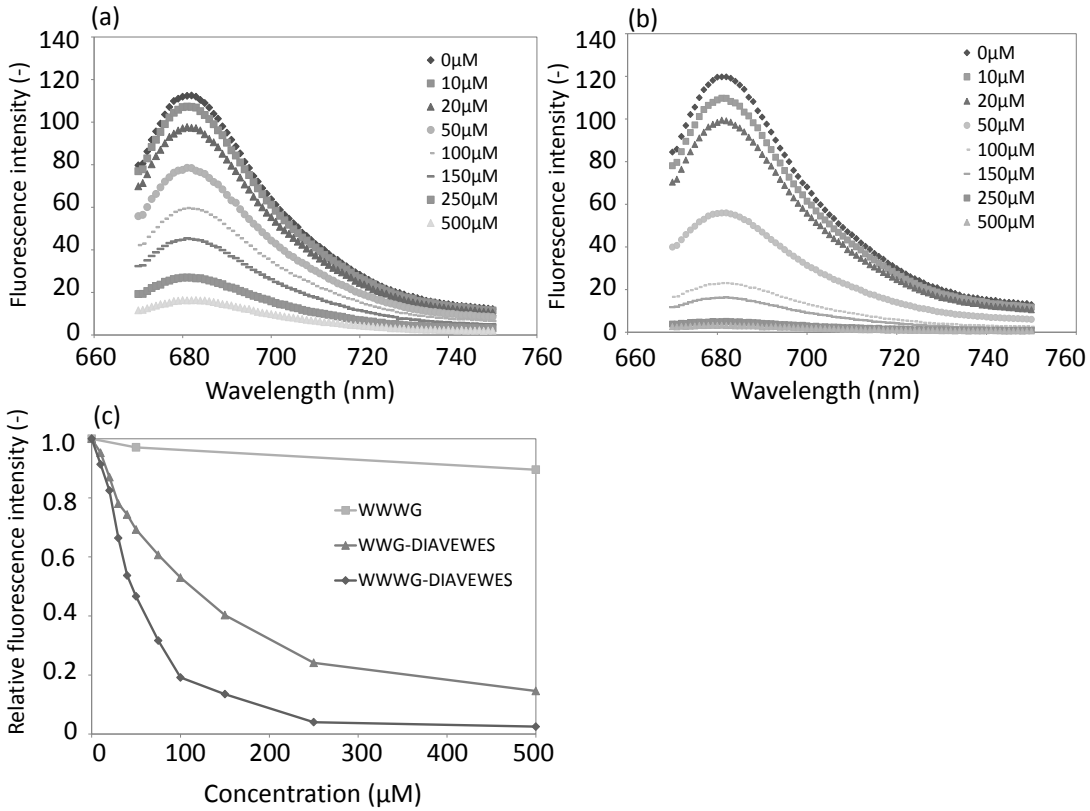

Fig. 3 Dose effect of quenching activity. (a) The spectrum of Atto655-labeled NKFRGKYK after the addition of WWG-DIAVEWES. (b) The spectrum of Atto655-labeled NKFRGKYK after the addition of WWWG-DIAVEWES. (c) The plots of fluorescence intensity at 684 nm versus the quenching peptide concentrations.

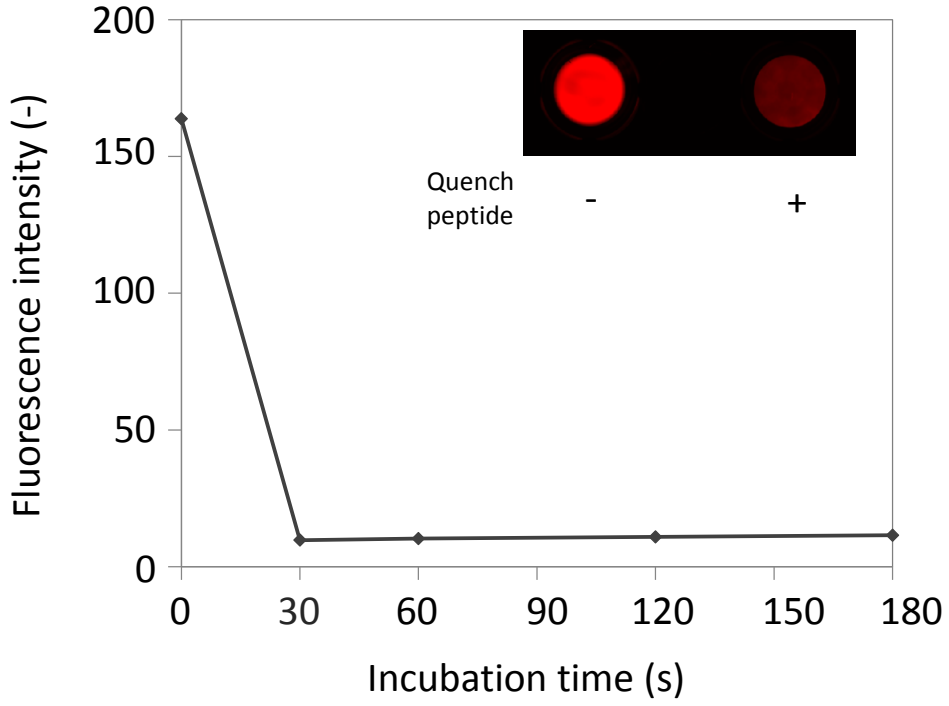

Fig. 4 Time course of fluorescence intensity after the addition of WWWG-DIAVEWES into Atto655-labeled NKFRGKYK peptide solution.

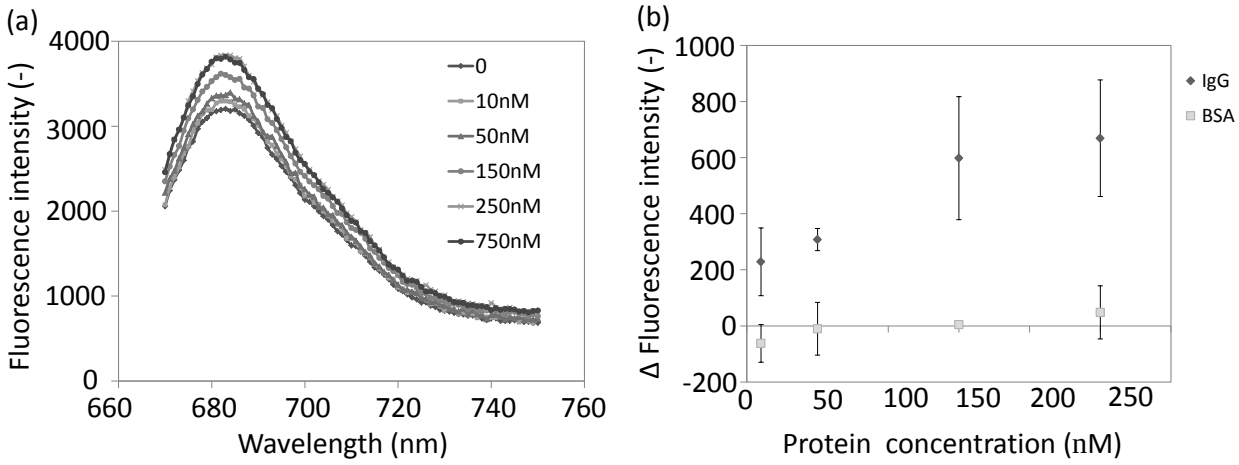

Fig. 5 IgG detection using Atto655-labeled NKFRGKYK and WWWG-DIAVEWES. Atto655-labeled NKFRGKYK and human IgG in PBS were pre-incubated for 10 min before adding WWWG-DIAVEWES. (a) The spectrum of Atto655-labeled NKFRGKYK after the addition of WWWG-DIAVEWES. (b) The plots of fluorescence intensity of Atto655-labeled NKFRGKYK versus protein concentration.

3.4. Summary

In this study, we proposed protein detection system using two peptides; molecular recognition peptide and quenching peptide incorporating tryptophan. IgG was used as the model protein and the Atto655-labeled NKFRGKYK was selected as the probe for recognition of IgG. The quenching peptide, WWWG-DIAVEWES, was newly designed using peptide array. The quenching peptide showed effective quenching of Atto655-labeled NKFRGKYK within 30 seconds. The quenching effect decreased in the presence of IgG, and IgG was detected above 10 nM. Therefore, it was shown that this technique is a simple and useful detection method with a short reaction time.

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Chapter 4

Design of a peptide molecular beacon incorporating an IgG-Fc binding peptide with its complementary quenching peptide

4.1. Introduction

The development of simple, rapid, sensitive, and inexpensive bioassays is of great importance for the detection of biomolecules. Conventional biological immunoassays have required relatively long reaction times and sequential washing steps, since most of these assays are performed in solid-phase [1-3]. Therefore, homogeneous assays that can detect target proteins easily and rapidly have been proposed as a powerful tool.

Fluorescence-based assays, such as fluorescence polarization and fluorescence energy transfer, which do not require separation steps, are advantageous due to their short assay time and ease of handling [4, 5]; a number of fluorescence-based homogeneous detection sensors have been reported [6-8]. Peptide beacons that are

conjugated with various fluorophores at the N-, or C-terminus have also attracted increasing attention as homogeneous assays [9-12]. The concept of peptide beacons has been reported by Wei *et al.* [13]. In the absence of a target protein, the peptide beacon forms a closed structure due to the interaction between the fluorophore and quencher, and theminimum fluorescence can be obtained. In the presence of a target protein, this closed structure is loosened and a large increase in fluorescence emission is achieved. In previous studies, peptide beacons labeled with various fluorophores have been developed, and some targets such as antibodies and DNA were detected [14-18]. Oh *et al.* [14, 15] have reported p17 antibody detection using a hexamer peptide beacon conjugated with pyrene or ruthenium (II) bisbipyridine-phenan-throline. Wu *et al.* [18] have reported DNA detection using a DNA binding peptide beacon conjugated with pyrene. Such peptide beacons only show recognition ability for target molecules. However, these peptide beacons required uncommon dyes that form duplexes, such as pyrene, or long lifetime dyes, such as ruthenium (II) bisbipyridine-phenan-throline. As a result, the stability of the closed structures of such peptide beacons has been relatively low. To overcome this disadvantage, some peptide beacons incorporating peptide nucleic acid (PNA) have been reported [19, 20]. However, such peptide beacons are complex and synthesis is time consuming. Moreover, non-specific protein binding could be induced by hydrophobic interactions with PNA.

Our research group has designed various peptides that interact with proteins, cell receptors, other peptides, and metal nanoparticles using peptide arrays [21-25], and has researched target detection using these peptides. In a previous study, an IgG-Fc high binding peptide (NKFRGKYK) and its complementary peptide (DIAVEWES) were designed from IgG Fcγ receptors and IgG-Fc, respectively [26, Sugita *et al.* in press],

and IgG was detected using two peptide probes, Atto655-labeled NKFRGKYK and WWWG-DIAVEWEW, which could induce quenching of Atto655 by photoinduced electron transfer (PeT) [27, 28]. However, since the dissociation constant between Atto655-labeled NKFRGKYK and WWWG-DIAVEWES was not high (1.2×10^{-5} M), a high concentration of WWWG-DIAVEWES was required to quench Atto655-labeled NKFRGKYK.

In this study, a peptide beacon was synthesized by connecting Atto655-labeled NKFRGKYK and DIAVEWES that conjugated tryptophan residues to the C-terminus, in order to form stable closed structures and induce efficient quenching. Using the peptide beacon, detection of IgG was investigated in a homogeneous assay. As far as we know, our peptide beacon designed with a fluorophore-labeled recognition peptide and a complementary quencher peptide is the first such molecule constructed for target protein detection without enzyme activity

4.2. Materials and methods

4.2.1. Peptide array synthesis

A cellulose membrane (grade 542; Whatman, Maidstone, UK) was activated using β-alanine as the N-terminal basal spacer. Fmoc-11-aminoundecanoic acid (Watanabe Chemical Inc., Hiroshima, Japan) was conjugated as an additional spacer between the candidate peptides and the cellulose. Activated Fmoc amino acids (0.25 M) were spotted to the membrane using a peptide auto-spotter (ASP222; Intavis AG, Köln, Germany) in accordance with the manufacturer's instructions with some modifications. A carboxyl group of activated Fmoc amino acid was combined with

11-aminoundecanoic acid on the cellulose membrane. After the first residue addition, the remaining amino groups were blocked using 5% acetic anhydride for 30 min. With each elongation step, the membrane was deprotected with 20% piperidine and subsequently washed thoroughly with N, N-dimethylformamide (DMF), followed by methanol. After the final deprotection, side-chain protecting groups were removed with a solution of m-cresol : thioanisol : ethandithiol : trifluoroacetic acid (1 : 6 : 3 : 40) for 3 h. Finally, the membrane was thoroughly washed with diethyl ether, methanol, and phosphate buffered saline (PBS, pH 7.4), respectively, and dried for the subsequent assay.

4.2.2. Design of quenching peptides on peptide arrays

To design complementary quenching peptides, the complementary peptide DIAVEWES was modified with tryptophan residues. The peptides were synthesized on the cellulose membrane using the peptide array. Each peptide spot was punched out and embedded into a 96-well plate. Peptide arrays were soaked in 1% Block Ace® (DS Pharma Biomedical Co., Ltd., Osaka, Japan) for 1 h at 37°C. After blocking, the arrays were washed three times by T-PBS and were incubated with Atto655-labeled NKFRGKYK solution (1 μM in PBS) for 1 h at 37°C in the dark. Fluorescence spectra of the peptide spots were measured using a fluorescence spectral reader (POWER SCAN 4, DS Pharma Biomedical Co., Ltd., Osaka, Japan) at 650 nm excitation.

4.2.3. Measurement of fluorescence spectra of the peptide beacon in various buffers

The peptide beacon (Atto655-labeled

NKFRGKYKGGSGGSDIAVEWESGWWWW) was purchased from Sigma-Aldrich Japan. This stock of peptide beacon was diluted to 10 μM in PBS (pH 7.4) with 10% DMSO. The peptide beacon solution was mixed with various PBS buffers in a cuvette, and the concentration of peptide beacon was further diluted to 100 nM. The fluorescence emission spectrum of the peptide beacon solution was recorded from 670 nm to 750 nm at room temperature using a spectrofluorophotometer (RF-5300PC, Shimadzu Corporation, Kyoto, Japan) at 650 nm excitation.

4.2.4. IgG detection using the peptide beacon

The 10 μM peptide beacon solution was diluted in PBS (pH 2.0). IgG (Sigma-Aldrich Japan, Tokyo, Japan) or BSA (Wako Pure Chemical Industries, Ltd., Osaka, Japan) solution in PBS (pH 7.4) was mixed with the peptide beacon solution, and the final concentration of peptide beacon and IgG was diluted to 100 nM and 0-10 μM, respectively. Mixing volume rates between IgG solution and the peptide beacon solution were controlled to pH conditions of pH 3.0, 4.0, 6.0, and 7.4. The mixture solutions were incubated for 60 min at 37 ˚C in the dark. The fluorescence emission spectrum of the peptide beacon solution was recorded from 670 nm to 750 nm at room temperature using a spectrofluorophotometer at 650 nm excitation.

In an IgG detection assay using a pH trigger, 10 μM peptide beacon solution was diluted at 150 nM in 0.10% T-PBS (pH 2.0). IgG or BSA solution in PBS (pH7.4) was mixed with the peptide beacon solution at a volume ratio of 1 : 2, and the final concentration of peptide beacon and IgG was diluted to be 100 nM and 0-10 μM, respectively, in the solution (0.05% T-PBS, pH 3.0). The mixture solutions were incubated for 60 min at 37˚C in the dark. After incubation, 1.0 M Tris buffer (pH 9.0)

was added to neutralize the mixture solution, and fluorescence emission spectra of the peptide beacon solutions were measured.

4.3. Results and discussion

4.3.1. Optimization of the complementary quenching peptide

In order to investigate the optimal number of tryptophan residues for quenching Atto655, complementary quenching peptides incorporating tryptophan residues at the C-terminus of DIAVEWES were synthesized on a peptide array. A glycine residue was inserted as a spacer between DIAVEWES and the tryptophan residues.

Fluorescence spectra of Atto655-labeled NKFRGKYK were measured using a fluorescence plate reader following 60 min incubation with the designed quenching peptide (Tryptophan-conjugated DIAVEWES). As the number of incorporated tryptophan residues increased, the decrease in fluorescence of Atto655-labeled NKFRGKYK was detected within all ranges from 660 nm to 750 nm. In contrast, only a small fluorescence decrease was observed in the case of alanine octameric peptide (poly A) incorporating tryptophan residues (Fig. 1a, -b). When Atto655-labeled NKFRGKYK peptides were incubated with the poly A incorporating pentameric tryptophan, a non-specific fluorescence decrease was observed (approximately 20%). This suggests that the tryptophan pentamer can quench Atto655 fluorescence via non-specific interactions. To minimize non-specific signal decrease, the complementary quenching peptide, DIAVEWES-GWWWW, was subjected to the next experiment.

4.3.2. Effect of buffer condition on the fluorescence spectra of peptide beacons

Peptide beacons that conjugated Atto655-labeld NKFRGKYK and DIAVEWESGWWWW with a $(GGS)_2$ linker were synthesized. In order to confirm a quenching effect without the addition of target IgG, the fluorescence spectrum was compared to that of Atto655-labeld NKFRGKYK (without a quenching peptide sequence). The fluorescence spectrum of the peptide beacon significantly decreased compared to that of Atto655-labeled NKFRGKYK (Fig. 2). This suggests that effective PeT quenching occurs and tryptophan residues exist in the proximity of Atto655 by the interaction of NKFRGKYK and DIAVEWES. The structure of the peptide beacon (NKFRGKYKGGSGGSDIAVEWES) was predicted using PEP-FOLD (an online resource for de novo peptide structure prediction) (Supplementary Fig. 1). It was also assumed that the peptide beacon formed a closed stem-loop structure in PBS at pH 7.4.

For detection of target proteins using peptide beacons, intramolecular interactions between the target protein recognition site (NKFRGKYK) and its complementary sequence (DIAVEWES) should be decreased so as to form an open structure for target binding. Peptide beacons were dissolved in PBS buffer containing HCl, tween 20 or NaCl. No increase in fluorescence intensity was observed in PBS containing tween or NaCl or acidic PBS (Supplementary Fig. 2). In contrast, an increase in the fluorescence spectrum was observed in the case of PBS containing tween (T-PBS) under acidic conditions. When the peptide beacon was dissolved in T-PBS containing 0.2% tween 20 at pH 2.0, a high fluorescence intensity was observed (Fig. 3a), and this level was almost the same intensity as that of Atto655-labeled NKFRGKYK (Fig. 2). These results indicate that the peptide beacon had changed its conformation to an open structure under this condition. The fluorescence intensity of the peptide beacon at pH 4.0 decreased approximately 50% compared with the levels recorded at pH 2.0 (Fig. 3b),

and a further decrease was observed at pH 6.0 and pH 7.4 (Figs. 3c and 3d).

The IgG-Fc binding peptide, NKFRGKYK, contains four positively charged residues of lysine (K) and arginine (R). In the complementary peptide sequence, DIAVEWES, there are three negatively charged residues of aspartic acid (D) and glutamic acid (E). Therefore, it is likely that the peptide beacon forms a stable closed structure as a result of electrostatic interaction. However, an increase in fluorescence intensity was not observed in PBS at pH 2.0. Since an increase in fluorescence intensity was observed when tween was added to PBS at pH 2.0 and pH4.0, our results also indicated that hydrophobic interactions between NKFRGKYK and DIAVEWES additionally contribute to the stability of the closed structure.

4.3.3. Effect of pH on the fluorescence intensity of the peptide beacon for IgG detection

We investigated IgG detection using the peptide beacon designed in the present study. The peptide beacon was incubated with IgG at various pH levels. Changes in fluorescence intensity of the peptide beacon were not observed when the peptide beacon was incubated with IgG in PBS (pH 7.4), PBS with added NaCl (pH 7.4), T-PBS (pH 7.4) or T-PBS with added NaCl (data not shown). In contrast, an increase in fluorescence intensity was observed in PBS at pH 3.0 and pH 4.0 (Figs. 4a and 4b). Only a slight increase in fluorescence intensity was observed in PBS at pH 5.0 and 6.0 (Figs. 4c and 4d).

IgG dose dependency was investigated in PBS at pH 3.0. The fluorescence intensity of the peptide beacon increased alongside increasing concentrations of IgG (Supplementary Fig. 3a). Supplementary Figure 3b shows the relationship between IgG

concentration and the fluorescence intensity at 684 nm, which is the fluorescence maximum of Atto655 dye. As a result, the increase in the fluorescence intensity of the peptide beacon was observed above 50 nM, and the fluorescence intensity increased to 300 AU at 2.5 μM IgG. When BSA was added to the peptide beacon instead of IgG, there was only a slight increase in fluorescence. However, the fluorescence intensity of the peptide beacon was comparable below 250 nM. These results suggest that non-specific interactions with BSA also induced a conformational change of the peptide beacon as it was also observed with tween in Fig. 3.

In order to minimize non-specific binding with BSA, the peptide beacon was dissolved in 0.1% tween containing PBS and sensitive detection of IgG was investigated. The peptide beacon was mixed with the sample solution containing IgG at pH 3.0. After incubating the peptide beacon with IgG for 60 min, the mixture was neutralized by adding 1.0 M Tris buffer (pH 9.0) to form a closed structure, since the peptide beacon forms an open structure in acidic T-PBS (Figs. 3a and 3b). Figure 5 shows the effect of IgG concentration on fluorescence intensity after neutralization. The fluorescence intensity of the peptide beacon without neutralization shows a maximum intensity above 400 AU, while a decrease in the fluorescence intensity of the peptide beacon was observed in a dose dependent manner with IgG after neutralization (Fig. 5a). In contrast, little change in the fluorescence intensity of the peptide beacon was observed with BSA. This suggests that non-specific interaction was avoided in the presence of tween. Figure 5b shows the relationship between the fluorescence intensity at 684 nm and IgG or BSA concentration. In contrast to Fig. 5b, specific detection of IgG was observed, and the difference in the fluorescence intensity between IgG and BSA was observed above 50 nM protein concentration (Fig. 5b). In order to confirm a clear timescale needed for IgG

detection, fluorescence intensity on a time series of IgG and peptide beacon interactions was investigated. The peptide beacon and IgG were incubated at pH 3.0, and neutralized by 1.0 M Tris buffer at pH 9.0. The relative fluorescence intensity increased with the duration of interaction time between IgG and the peptide beacon, reaching saturation after 60 min (Fig. 6). Therefore, we demonstrated that specific detection of IgG could be measured using this peptide beacon above an IgG concentration of 50 nM for 60 min.

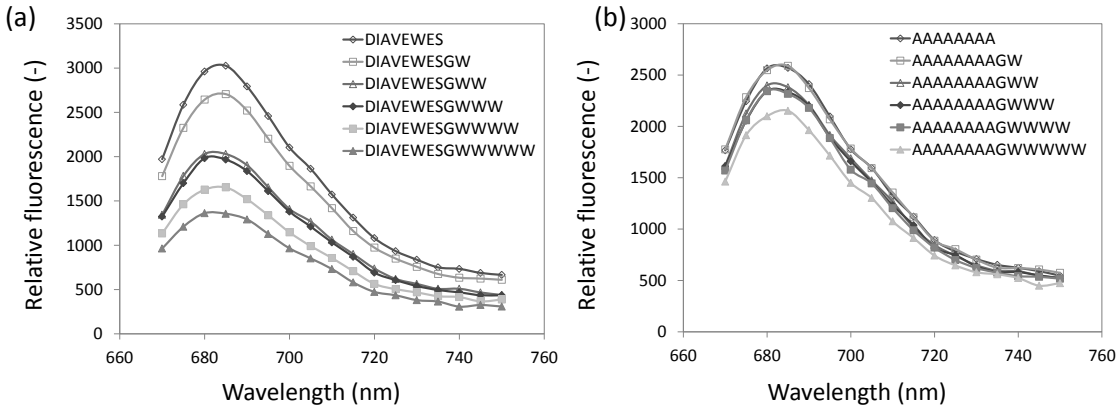


Fig. 1 Quenching effect of tryptophan-conjugated complementary peptides on a peptide array.

Fluorescence spectra of Atto655-labeled NKFRGKYK were measured using a fluorescence reader following incubation for 60 min on a peptide array; DIAVEWES (a) and AAAAAAAA (b) conjugated with tryptophan residues at the C-terminus.

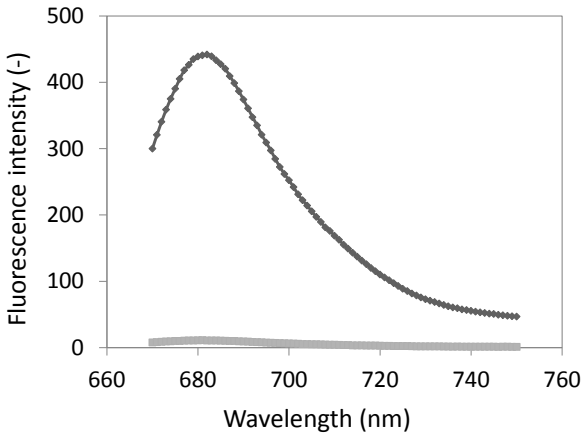
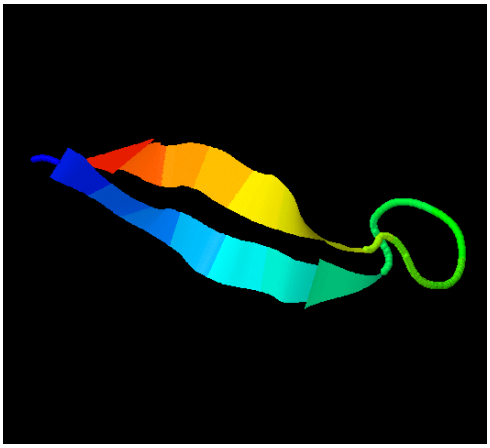


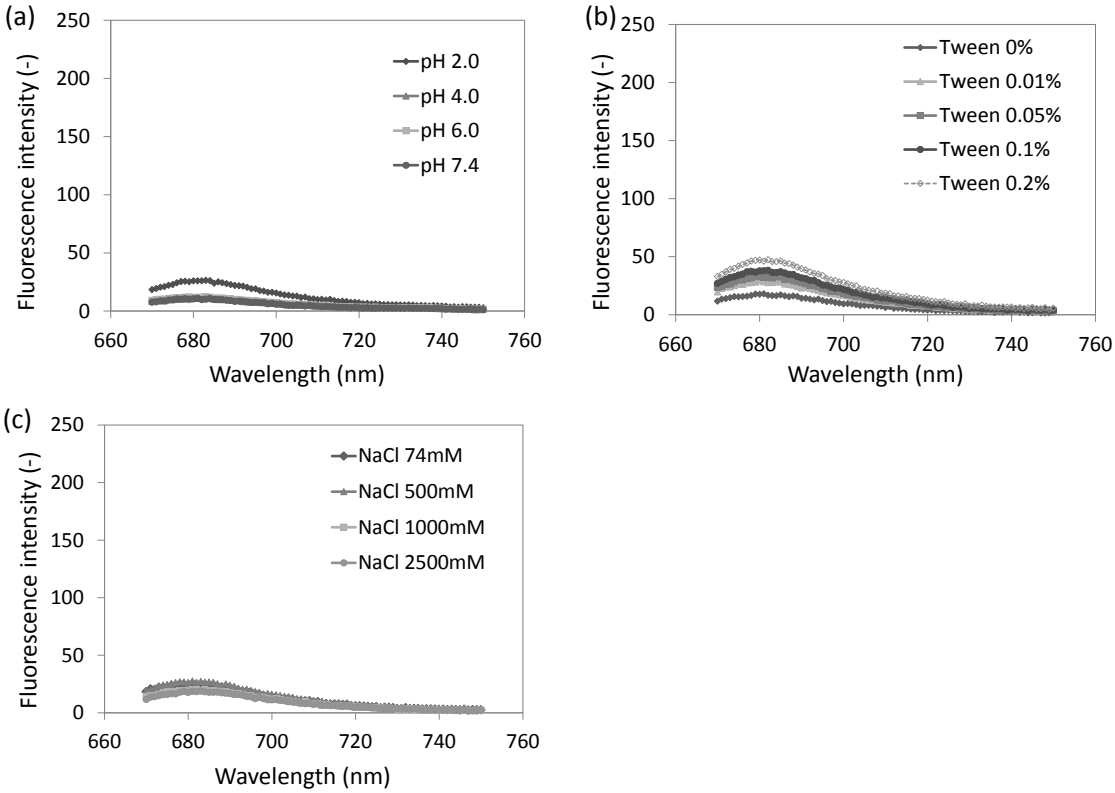
Fig. 2 Comparison of fluorescence spectra between Atto655-labeled NKFRGKYK and the peptide beacon.

The peptides were dissolved to 10 μM in PBS (pH 7.4) with 10% DMSO, and diluted to 100 nM with PBS (pH 7.4). Black rhombus line: Atto655-labeled NKFRGKYK. Gray square line: the peptide beacon (Atto655-NKFRGKYK-GGSGGS-DIAVEWES-GWWWW).



Supplementary Fig. 1 Folding model of the peptide beacon.

The structure of the peptide beacon (NKFRGKYKGGSGGSDIAVEWES) was predicted using PEP-FOLD (an online resource for *de novo* peptide structure prediction). The blue cartoon shows NKFRGKYK. Green loop shows $(GGS)_2$ linker. The yellow cartoon shows DIAVESES.



Supplementary Fig. 2 Fluorescence spectra of the peptide beacon dissolved in various buffers.

(a) in PBS (pH 2.0, 4.0, 6.0, 7.4), (b) in T-PBS (pH 7.4), (c) in PBS with added NaCl (pH7.4).

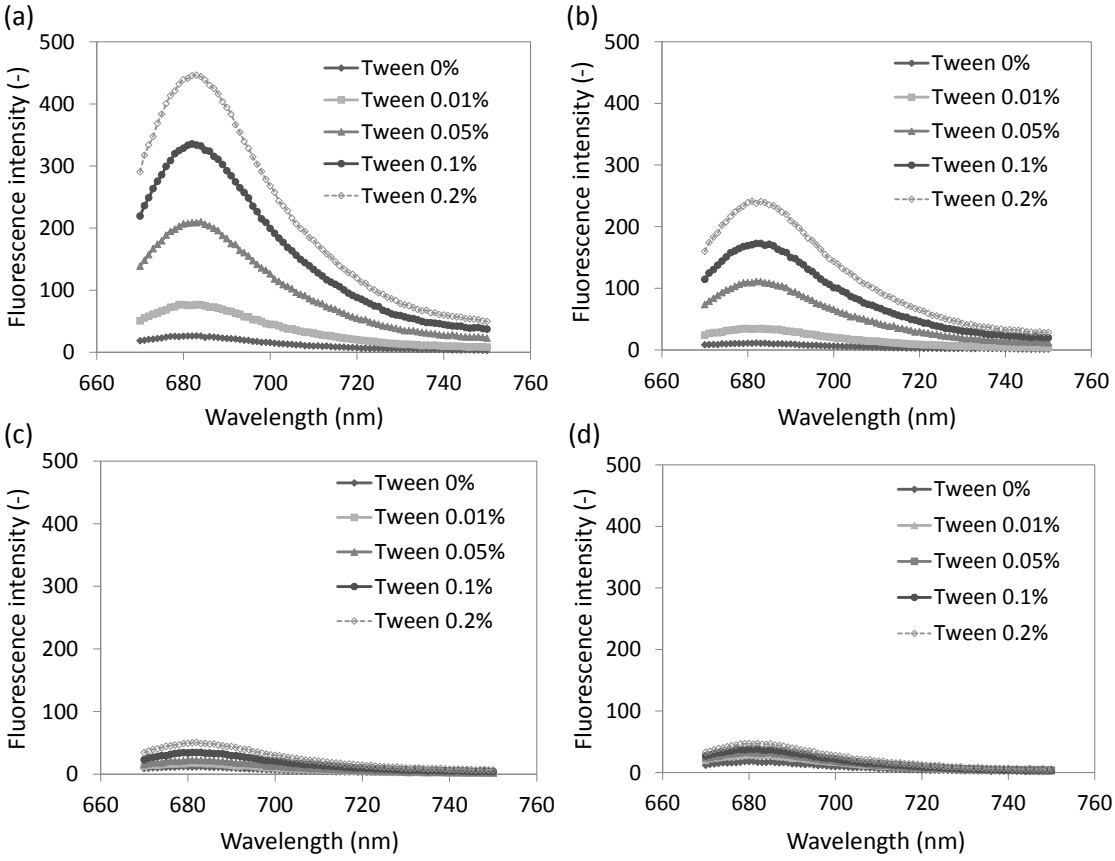


Fig. 3 The effect of pH on the fluorescence spectrum of the peptide beacon.

Peptide beacon solution (10 μM) was mixed with various T-PBS (tween 20 concentration of 0-0.2%) at a volume ratio of 1:99 (final peptide concentration: 100nM). (a) pH 2.0, (b) pH 4.0, (c) pH 6.0, (d) pH 7.4.

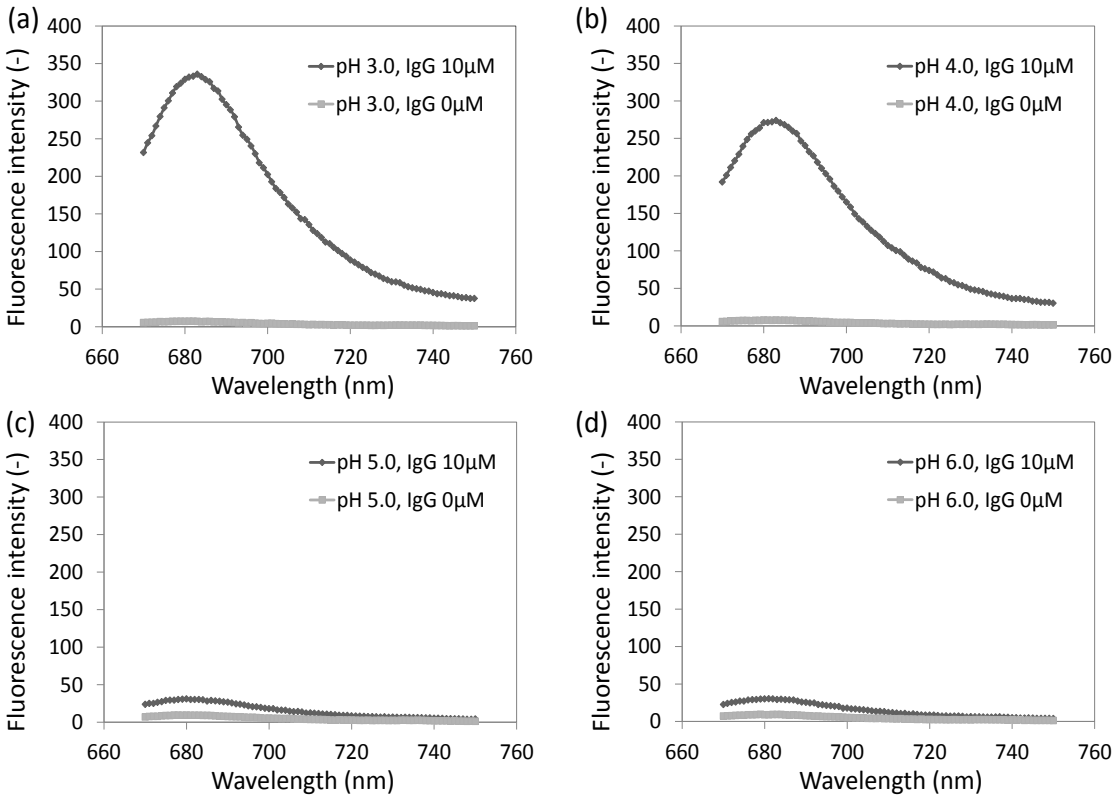
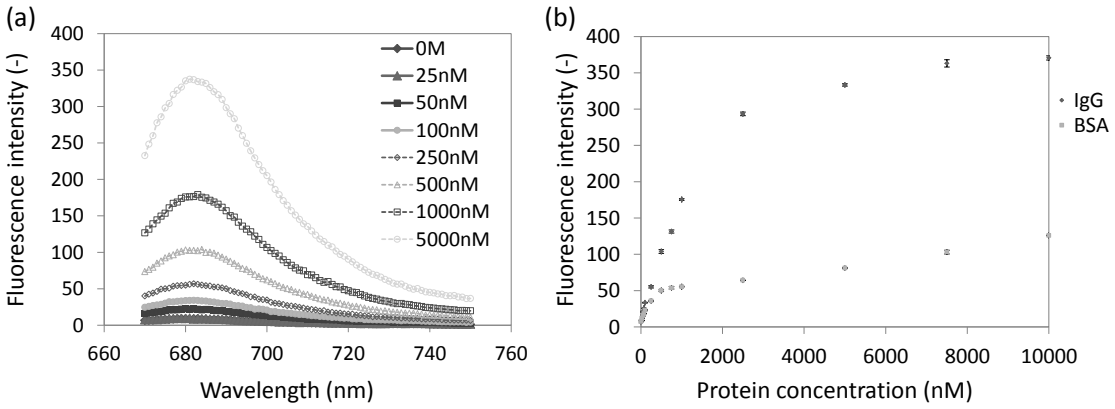


Fig. 4 The effect of pH on fluorescence intensity during the detection of IgG using the peptide beacon.

IgG solution in PBS (pH7.4) was mixed with peptide beacon solution (pH 2.0). Mixture volume ratios between IgG solution and peptide beacon solution were controlled to achieve a final concentration of 100 nM peptide and 10 μM IgG at various pH, pH 3.0 (a), pH 4.0 (b), pH 5.0 (c), pH 6.0 (d).



Supplementary Fig. 3 Detection of IgG using the peptide beacon at pH 3.0.

(a) Fluorescence spectra of the peptide beacon solution with various concentration of IgG, (b) The effect of IgG and BSA concentration on fluorescence intensity of the peptide beacon, with excitation at 650 nm and emission at 684 nm.

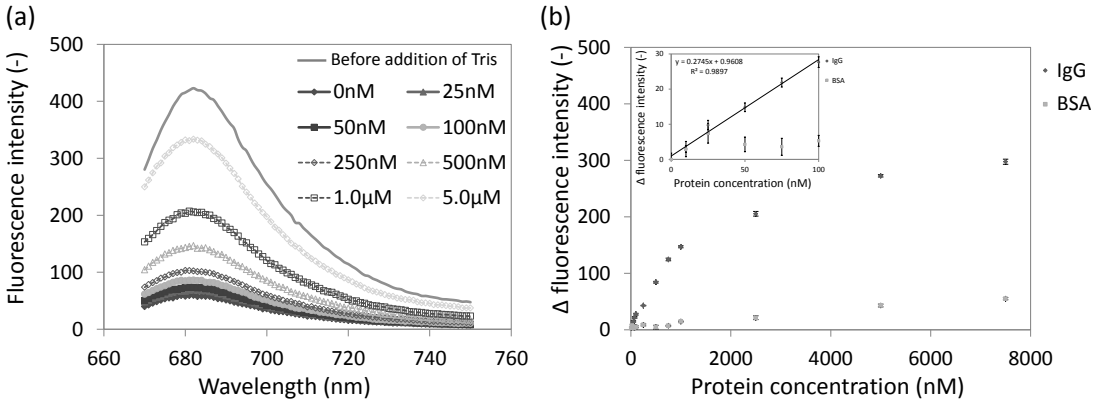


Fig. 5 The pH-triggered detection of IgG using the peptide beacon.

(a) Fluorescence spectra of the peptide beacon at various concentrations of IgG. The peptide beacon solution was mixed in various concentration of IgG and incubated for 60 min before being neutralized.

(b) Effect of IgG (dark gray circle) and BSA (light gray circle) concentration on the relative fluorescence intensity of the peptide beacon. The fluorescence intensity of the peptide beacon without IgG or BSA was subtracted.

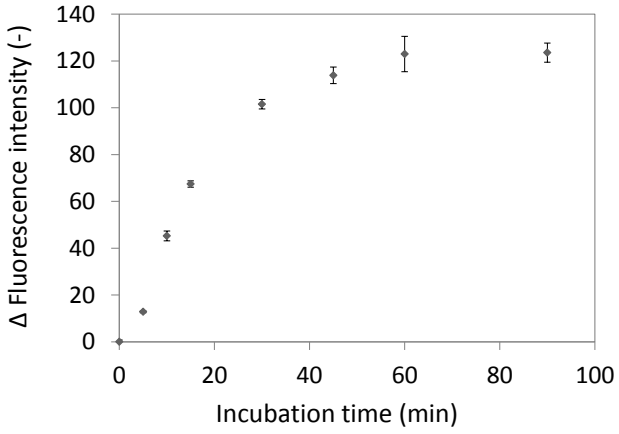


Fig. 6 A time series of IgG and peptide beacon interaction and the effects on the relative fluorescence intensity at 684 nm. IgG solution (final concentration: 1.0 μM) was mixed with peptide beacon solution (final concentration: 100 nM) at pH 3.0 and subsequently neutralized.

4.4. Summary

A fluorophore-labeled peptide beacon, which forms a closed structure by intramolecular interaction, was designed to detect target proteins in a homogeneous assay. An Atto655-labeled NKFRGKYK peptide, which could bind to the Fc region of IgG, was conjugated with its complementary quenching peptide (DIAVEWES-GWWWW) via a $(GGS)_2$ linker. In the absence of IgG, the peptide beacon formed a closed stem-loop-like structure due to the interaction between NKFRGKYK and DIAVEWES, and the quenching of the fluorescence signal of Atto655 by tryptophan residues was observed. In the presence of IgG, the beacon remained an open structure due to the recognition of NKFRGKYK to IgG, and the fluorescence of Atto655 was detected. The fluorescence intensity of the peptide beacon increased alongside the concentration of IgG, and the detection limit of IgG was 50 nM.

4.5. References

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Chapter 5

Concluding remarks

Antibodies have been attracted as molecules for medical diagnostics, drug and detection of various targets. In this thesis, development of peptide ligands for antibody purification and homogeneous IgG detection methods for diagnostics were aimed.

In the Chapter 1, a general introduction covering the importance of antibody researches and applications of peptides in antibody therapy and antibody detection were discussed. Considering to these backgrounds, the objective of this thesis was also described.

In the Chapter 2, screening of the IgG-Fc binding peptides was investigated using a spot peptide array and its usefulness as the IgG purification ligand was evaluated. As the result of 3 steps screening, the two peptides, NKFRGKYK and NARKFYKG that have high binding affinity to IgG were obtained. These peptide ligands were used for the purification of an antibody from cell culture supernatants. The yields obtained for the antibody were found to be 69% and 80% respectively. The residual DNA and HCP reduction obtained by these peptides resin were in the range of 4.2 and 1.3LRV (NKFRGKYK), 3.9 and 1.5LRV (NARKFYKG) respectively. Therefore, it was

indicated that the screened NKFRGKYK and NARKFYKG peptide would be useful as affinity purification ligands for IgG.

In the Chapter 3, protein detection system using two peptides; molecular recognition peptide and quenching peptide incorporating tryptophan was developed. IgG was used as the model protein and the Atto655-labeled NKFRGKYK was selected as the probe for recognition of IgG. The quenching peptide, WWWG-DIAVEWES, was newly designed using peptide array. The quenching peptide showed effective quenching of Atto655-labeled NKFRGKYK within 30 seconds. The quenching effect decreased in the presence of IgG, and IgG was detected above 10 nM. Therefore, it was shown that this technique is a simple and useful detection method with a short reaction time.

In the Chapter 4, a fluorophore-labeled peptide beacon, which forms a closed structure by intramolecular interaction, was designed to detect target proteins in a homogeneous assay. An atto655-labeled NKFRGKYK peptide, which could bind to the Fc region of IgG, was conjugated with its complementary quenching peptide (DIAVEWES-GWWWW) via a $(GGS)_2$ linker. In the absence of IgG, the peptide beacon formed a closed stem-loop-like structure due to the interaction between NKFRGKYK and DIAVEWES, and the quenching of the fluorescence signal of Atto655 by tryptophan residues was observed. In the presence of IgG, the beacon remained an open structure due to the recognition of NKFRGKYK to IgG, and the fluorescence of Atto655 was detected. The fluorescence intensity of the peptide beacon increased alongside the concentration of IgG, and the detection limit of IgG was 50 nM. Therefore, we demonstrated that our concept of a peptide beacon was useful for protein

detection.

In this thesis, peptide ligands, NKFRGKYK and NARKFYKG, for antibody purification were screened (Chapter 2), and homogeneous IgG detection methods were developed using IgG binding peptide (NKFRGKYK) and its complementary peptides (DIAVEWES) (Chapter 3, 4). These peptides and methods were useful for antibody purification and antibody detection.

I hope that the peptide ligands will be contribute to reduction of an antibody production cost, and the homogeneous antibody detection methods will be applied to diagnostic tool.

List of publications for dissertation

1. **Tomoya Sugita**, Makoto Katayama, Mina Okochi, Ryuji Kato, Takamitsu Ichihara and Hiroyuki Honda: Screening of peptide ligands that bind to the Fc region of IgG using peptide array and its application to affinity purification of antibody, Biochem. Eng. J., 79 (4), 33-40, 2013

2. **Tomoya Sugita**, Mina Okochi, and Hiroyuki Honda: Design of quenching peptide probes incorporating tryptophan for rapid antibody detection, Chem. Lett., in press, 2014

3. **Tomoya Sugita**, Mina Okochi, and Hiroyuki Honda: Design of a peptide beacon incorporating an IgG-Fc binding peptide with its complementary quenching peptide, Submitted

Other publications

1. Mina Okochi, **Tomoya Sugita**, Seiji Furusawa, Mitsuo Umetsu, Tadafumi Adschiri and Hiroyuki Honda: Peptide array-based characterization and design of ZnO-high affinity peptides, Biotechnol. Bioeng., 106 (6), 845-51, 2010

2. Mina Okochi, Masafumi Ogawa, Chiaki Kaga, **Tomoya Sugita**, Yasuyuki Tomita, Ryuji Kato and Hiroyuki Honda: Screening of peptides with a high affinity for ZnO using spot-synthesized peptide arrays and computational analysis, Acta. Biomater., 6, 2301-2306, 2010

3. Makoto Katayama, **Tomoya Sugita**, Ryuji Kato, Mina Okochi, Miyoko Matsushima, Tsutomu Kawabe, Tomokazu Takase, Yasuko Yoshida, Mitsuo Kawase and Hiroyuki Honda: Screening of IgG-Fc binding peptides from milk protein using slide glass type-exclusive peptide array, Kagakukougaku Ronbunshu, 37, 546-550, 2011

4. Yuji Asai, **Tomoya Sugita**, Ryuji Kato, Mina Okochi, Kyuya Nakagawa and Hiroyuki Honda: Enhancement of the Activity of a Lactobacilli-Aggregating Peptide by Freezing Treatment, J. Chem. Eng. Jpn., 45 (8), 609-614, 2012

5. Takashi Ochiai, **Tomoya Sugita**, Ryuji Kato, Mina Okochi and Hiroyuki Honda: Screening of an α-Amylase Inhibitor Peptide by Photolinker-Peptide Array, Biosci. Biotechnol. Biochem., 76 (4), 819-24, 2012

Conferences

International 2 times, Domestic 12 times

(The conferences related this thesis are described below)

1. **Tomoya Sugita**, Makoto Katayama, Ryuji Kato, Mina Okochi and Hiroyuki Honda: Screening of peptide ligands that bind to the Fc region of IgG using peptide array, The Society of Chemical Engineers 77th Annual Meeting, Tokyo, Japan, March, 2012
2. **Tomoya Sugita**, Tomohiro Kamiya, Mina Okochi and Hiroyuki Honda: Development of IgG homogeneous detection using IgG binding peptide, The Society of Chemical Engineers 44th Autumn Meeting, Tokyo, Japan, September, 2012
3. Tomoya Tanabe, **Tomoya Sugita,** Mina Okochi and Hiroyuki Honda: Research of the IgG selective peptide ligands for antibody purification, The Society of Chemical Engineers 78th Annual Meeting, Tokyo, Japan, March, 2013
4. **Tomoya Sugita**, Mina Okochi and Hiroyuki Honda: Design of quench peptide probe incorporated tryptophan for antibody detection, The Society of Chemical Engineers 78th Annual Meeting, Tokyo, Japan, March, 2013
5. **Tomoya Sugita**, Mina Okochi and Hiroyuki Honda: Screening of IgG binding peptide and its application to homogeneous IgG detection, The Chemical Society of Japan 7th Bio-related chemical symposium, Aichi, Japan, September, 2013

Other conferences

1. Mina Okochi, Seiji Furusawa, **Tomoya Sugita**, Mituo Umetsu, Masahumi Adschri, and Hiroyuki Honda: Peptide array-based characterization and design of ZnO-high affinity peptides, The Society of Chemical Engineers 38th Autumn Meeting, Fukuoka, Japan, September, 2006
2. **Tomoya Sugita**, Mina Okochi, Seiji Furusawa, Yasuyuki Tomita, Mituo Umetsu, Masahumi Adschri, and Hiroyuki Honda: Characterization of ZnO-high affinity peptides using peptide array, The Chemical Society of Japan 87th Annual Meeting, Osaka, Japan, March, 2007
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4. Mina Okochi, **Tomoya Sugita**, Hiroki Hayashi, and Hiroyuki Honda: Epitope mapping of antigen protein of *Heamophilus parainfluenzae* that related to IgA nephropathy using peptide array, The Society of Chemical Engineers 39th Autumn Meeting, Hokkaido, Japan, September, 2007
5. **Tomoya Sugita**, Mina Okochi, Hiroki Hayashi, and Hiroyuki Honda: "Screening of epitope peptides of antigen protein of *Heamophilus parainfluenzae* that related to IgA nephropathy using peptide array, The Chemical Society of Japan 88th Annual Meeting, Tokyo, Japan, March, 2008
6. Mina Okochi, **Tomoya Sugita**, Yuji Asai, and Hiroyuki Honda: Screening of peptides that control lactic acid bacterial adhesion and biofilm formation, The

Society for Biotechnology 61th Annual Meeting, Aichi, Japan, September, 2009

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8. Mina Okochi, Yuji Asai, **Tomoya Sugita**, and Hiroyuki Honda: Screening of peptides associated with adhesion and biofilm formation of lactic acid bacteria, American Society for Microbiology, San Francisco, California, 2012
9. **Tomoya Sugita**, Yuji Asai, Mina Okochi, and Hiroyuki Honda: Screening of peptides associated with adhesion of lactic acid bacteria and probiotic effect for intestinal cells, IGER International Symposium on Cell Surface Structures and Functions, Aichi, Japan, 2013

Patents

1. 特許出願番号 2009-189756 : 本多　裕之, 大河内　美奈, 杉田　智哉: 乳酸菌の付着性を誘導するペプチド及びその用途

2. 特許出願番号 2013-239263 : 本多　裕之, 大河内　美奈, 杉田　智哉　蛍光ラベルを行った F-ペプチドとクエンチャー（トリプトファン）ラベルした C-ペプチドによる抗体の定量検出

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