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Homogeneous One-Step Immunoassay Based on Switching Peptides for Detection of the Influenza Virus

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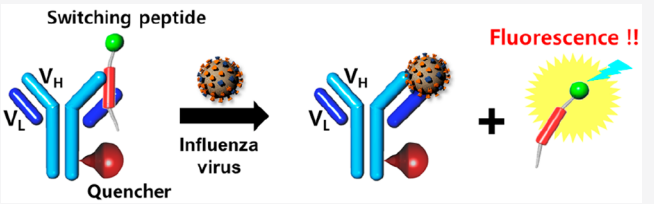
ABSTRACT: In this study, a homogeneous one-step immunoassay based on switching peptides is presented for the detection of influenza viruses A and B (Inf-A and Inf-B, respectively). The one-step immunoassay represents an immunoassay method that does not involve any washing steps, only treatment of the sample. In this method, fluorescence-labeled switching peptides quantitatively dissociate from the antigen-binding site of immunoglobulin G (IgG). In particular, the one-step immunoassay based on soluble detection antibodies with switching peptides is called a homogeneous one-step immunoassay. The immunoassay developed uses switching peptides labeled with two types of fluorescence dyes (FAM and TAMRA) and detection antibodies labeled with two types of fluorescence quenchers (TQ2 for FAM and TQ3 for TAMRA). The optimal switching peptides for the detection of Inf-A and Inf-B have been selected as L1-peptide and H2-peptide. The interactions between the four kinds of switching peptides and IgG have been analyzed using computational docking simulation and SPR biosensor. The location of labeling for the fluorescence quenchers has been determined based on the distance between the fluorescence dyes of the switching peptides and the fluorescence quenchers, calculated on the basis of the efficiency of fluorescence quenching, using the Förster equation. To demonstrate the feasibility of the one-step immunoassay, binding constants (K_D) have been calculated for detection antibodies against Inf-A and Inf-B with target antigens (Inf-A and Inf-B) and switching peptides (L1- and H2-peptides), using an isotherm model. The immunoassay has been demonstrated to be feasible using antigens as well as real samples of Inf-A and Inf-B with a critical cycle number (Ct). The immunoassay has also been compared to other commercially available rapid test kits for Inf-A and Inf-B and found to be far more sensitive for detection of Inf-A and Inf-B over the entire detection range.



Recently, switching peptides have been reported to bind reversibly at the binding pocket of immunoglobulin G (IgG), which is known to self-assemble, by means of interaction of the frame regions (FRs) of the heavy chain (V_H) and light chain (V_L).[1] Especially, the L1-region in the V_L interacts with the H2-region of V_H, and these regions are synthesized into L1- and H2-peptides, respectively.[2−5] Such switching peptides are known to bind specifically to the corresponding regions at the antigen-binding pocket of IgG: H2-peptide to the L1-region, and L1-peptide to the H2-region, as shown in Figure 1a. As these switching peptides labeled with fluorescence dyes could quantitatively dissociate from IgG according to the amount of bound antigen, a one-step immunoassay without any washing steps, but involving only treatment of the sample and measurement of fluorescence from the switching peptides, has been considered to be feasible. Such a one-step immunoassay has already been conducted using immobilized detection antibodies on solid supports, such as microplates and beads. In the case of inhomogeneous immunoassays using immobilized antibodies,[6,7] target analytes comparable to antibodies could be effectively bound to the antigen-binding pocket of IgG, with effective washing out of nonspecific binding.

Homogeneous immunoassays usually use free antibodies in solution (unimmobilized detection antibodies) for specific binding of target analytes.[6,7] For the detection of target analytes that are much larger in size than IgGs, such as bacteria, viruses, and parasites, a homogeneous immunoassay is required for the effective binding of antigens on the target analytes. In the case of immunosorbent assays, the binding pocket of antibodies are known to be only partially exposed for the binding of antigens and some studies reported that the proportion of active binding pocket of immobilized antibodies was estimated to be less than 20%.[8,9] Additionally, the number of antibodies bound to the solid supports were limited in comparison with the homogeneous immunoassays which used soluble antibodies in the assay medium. They usually have low immobilization efficiency due to steric hindrance.[10] These

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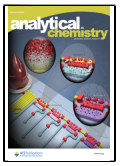

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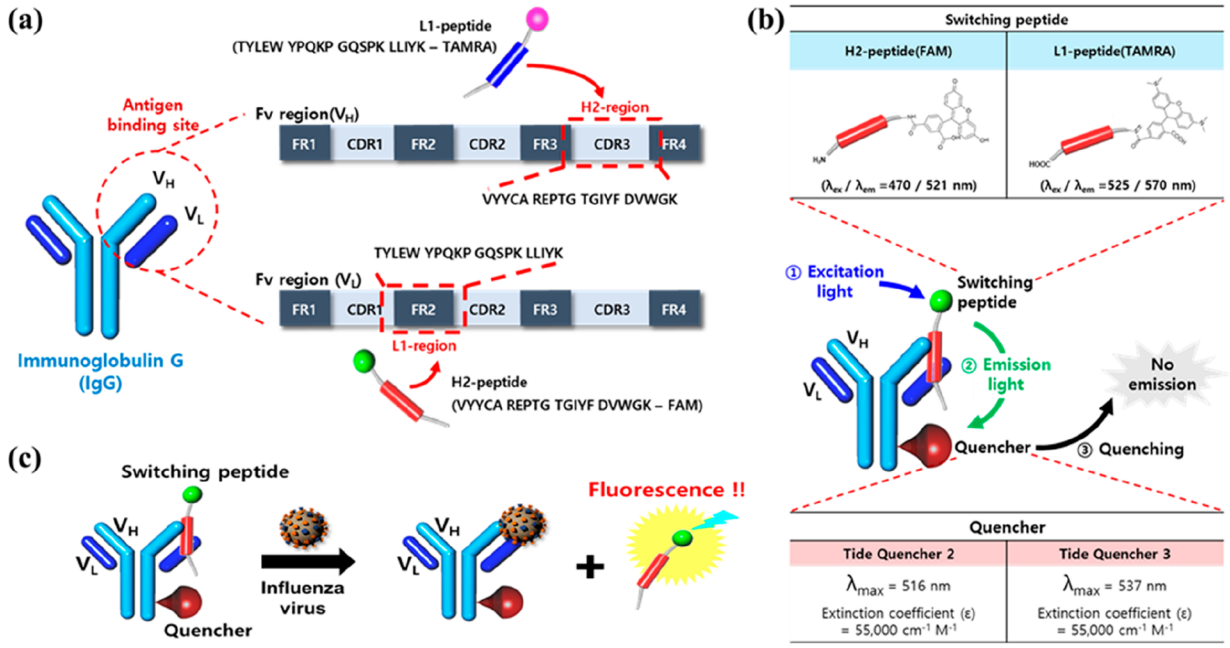

Figure 1. Principle of homogeneous one-step immunoassay. (a) Switching peptides that specifically binds to an antigen-binding pocket of IgG: H2-peptide to the L1-region, and L1-peptide to the H2-region. (b) The antibody labeled with fluorescence quencher for one-step immunoassay. (c) The homogeneous one-step immunoassay based on switching peptides for the detection of influenza virus.

limited number of antibodies coated on the solid support further restrict the performance of the assays. Most of all, the mass transport to the immobilized antibodies were diffusion-controlled with a restricted degree of freedom, resulting in a long incubation time for static equilibrium.[11] This diffusion process is the rate-determining step of the solid-phase kinetics, therefore, many researches utilize vortex agitation,[11] micro/nanoparticles[12] or porous matrix[13] to compress the antibodies in a diffusion area. In the homogeneous immunoassay, the concentration of antigens plays the most significant role that they do not in immunosorbent assay, resulting in the enhanced sensitivity and much faster reaction times.[14]

For the development of a homogeneous immunoassay using detection antibodies in solution in this study, a fluorescence quencher was labeled with antibodies, as shown in Figure 1b. Such a homogeneous immunoassay could be effectively used for the detection of analytes that are much larger in comparison to the detection antibodies, such as viruses and bacteria. In terms of the fluorescence dyes, the H2-peptide was labeled with FAM (λ_{ex} = 490 nm, λ_{em} = 521 nm),[15,16] while the L1-peptide was labeled with TAMRA (λ_{ex} = 525 nm, λ_{em} = 570 nm).[17,18] Commercially available fluorescence quenchers called Tide Quencher 2 (λ_{abs} = 516 nm) for FAM and Tide Quencher 3 (λ_{abs} = 573 nm) for TAMRA were labeled with the detection antibodies, in order to adsorb the fluorescence from the switching peptides.[19,20] Therefore, the detection antibodies (bound with switching peptides at the antigen-binding pocket) had no fluorescence in solution, even at the excitation wavelength of the fluorescence dye on the switching peptides. This homogeneous immunoassay based on switching peptides was then applied for the detection of Inf-A and Inf-B. As shown in Figure 1c, the samples positive for influenza virus resulted in the binding of the virus to the binding pocket of the detection antibodies. Subsequently, a quantitative amount of switching peptides was dissociated, and there was an increase in the fluorescence in the solution. In case of the negative sample without influenza virus, the detection antibodies produced no fluorescence signal before and even after sample treatment, due to the effect of the labeled fluorescence quencher. In this way, a homogeneous immunoassay could be realized even in solution, without binding the immobilizing antibodies to the switching peptides.

In this work, a homogeneous one-step immunoassay based on switching peptides has been presented for the detection of Inf-A and Inf-B. The homogeneous one-step immunoassay was prepared using switching peptides labeled with two types of fluorescence dyes (FAM and TAMRA) and detection antibodies labeled with two types of fluorescence quenchers (TQ2 for FAM and TQ3 for TAMRA). The optimal switching peptides for the detection of Inf-A and Inf-B were selected as L1-peptide and H2-peptide. The interactions between the four kinds of switching peptides and IgG have been analyzed using computational docking simulation and surface plasmon resonance (SPR) biosensor. The location of labeling for fluorescence quenchers was calculated based on the distance between the fluorescence dyes of the switching peptides and the fluorescence quenchers, which were calculated from the efficiency of fluorescence quenching, using the Förster equation. To demonstrate the feasibility of the one-step immunoassay, binding constants (K_D) were calculated for the Inf-A and Inf-B detection antibodies against target antigens (Inf-A and Inf-B) and switching peptides (L1- and H2-peptides), using an isotherm model. The homogeneous one-step immunoassay for Inf-A and Inf-B was demonstrated to be feasible using antigens, as well as, real samples of Inf-A and Inf-B, with a critical cycle number (Ct). Finally, the sensitivity of the homogeneous one-step immunoassay based on switching peptides was compared with that of conventional rapid tests for the detection of Inf-A and Inf-B.

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RESULTS AND DISCUSSION

Analysis of Fluorescence Quenching of Antibody Complex with Switching Peptides. In this study, two types of switching peptides were used for the detection of Inf-A and -B. These switching peptides were found to bind to the antigen-binding site of IgG by means of a computational docking simulation using PepATTRACT (https://bioserv.rpbs.univ-paris-diderot.fr/services/pepATTRACT/). As shown in Supporting Information (SI) Figure S1a, the L1 (and L2)-peptide bound to the H2 (and H1)-region of the heavy chain of IgG (V_H), while the H1 (and H2)-peptide bound to the L2 (and L1)-region at the light chain of IgG (V_L), respectively.[1] The four switching peptides were docked to the antigen-binding site of IgG (mouse Fab fragments, PDB ID = 1DQJ) using a computational docking simulation with the CABS-dock web server.[21,22] To analyze the binding of switching peptides to the binding pocket of IgG, the amino acid sequences of the CDR regions of the model antibody were taken from references 23−25. CDR1 (amino acid numbers: 31−35), CDR2 (amino acid numbers: 50−65), CDR3 (amino acid numbers: 95−102) at the V_H and CDR1 (amino acid numbers: 24−34), CDR2 (amino acid numbers: 50−56), and CDR3 (amino acid numbers: 89−97) at the V_L. From the computer simulation, the amino acids for the interaction between switching peptides and regions at antibodies were analyzed, which were the same as designed in Figure 1a. Additionally, the average distance from the amino acids for the interaction between switching peptides and the regions of the antibodies were estimated to be in the range of 2.5−3.5 Å. The interaction parameters between each switching peptide and the Fv-region of IgG are summarized in Table 1.

Table 1. Analysis of the Interaction between Switching Peptides and Amino Acids at the IgG Binding Pocket

	amino acids for interaction		
peptide name	switching peptide	antibody	distance (å)
H2-peptide	Tyr3	Ser22 (FR1, V_L)	3.15
	Thr11	Gly66 (FR3, V_L)	3.11
	Asp16	Ser30 (CDR1, V_L)	2.61, 3.12
	Val17	Tyr50 (CDR2, V_L)	3.19
	Trp18	Tyr50 (CDR2, V_L)	2.76
		Ser91 (CDR3, V_L)	2.69
L1-peptide	Leu3	Ser56 (CDR2, V_L)	3.09
	Tyr6	Val2 (FR1, V_H)	3.01
	Gln8	Ser97 (FR3, V_H)	2.90
L2-peptide	Val10	Tyr50 (FR2, V_L)	2.58, 3.01
H1-peptide	Trp3	Ser93 (CDR3, V_L)	3.03
	His5	Tyr58 (FR3, V_H)	3.45
	Gly19	Tyr33 (FR2, V_H)	3.38
	Glu20		3.55

For the homogeneous immunoassay using detection antibodies in solution (unimmobilized detection antibodies), a fluorescence quencher was labeled with antibodies, as shown in Figure 1b. The H2 (and L2)-peptide was labeled with FAM (λ_{ex} = 490 nm, λ_{em} = 521 nm) and L1 (and H1)-peptides were labeled with TAMRA (λ_{ex} = 525 nm, λ_{em} = 570 nm). Commercially available fluorescence quenchers called Tide Quencher 2 (λ_{abs} = 516 nm) for FAM and Tide Quencher 3 (λ_{abs} = 573 nm) for TAMRA were labeled with the detection antibodies, in order to adsorb the fluorescence from the switching peptides. For the homogeneous one-step immunoassay based on switching peptides and detection antibodies labeled with fluorescence quenchers, two important parameters were estimated: (1) the efficiency of fluorescence quenching in the one-step immunoassay complex of antibodies and switching peptides,[19,26,27] and (2) the binding sites of fluorescence quenchers at the detection antibodies, by means of measurement of FRET distance.[28−30]

The efficiency of fluorescence quenching was measured after the binding of switching peptides labeled with two kinds of fluorescence dyes (FAM and TAMRA) to the antibodies labeled with two kinds of fluorescence quenchers (TQ2 for FAM and TQ3 for TAMRA). As the size of the antigen-binding fragment of IgG is known to be within 2.5 nm in diameter (V_H domain),[31] the subtle difference in the location of the four kinds of switching peptides within the antigen-binding pocket of IgG was considered to make no significant difference in comparison to the location of the fluorescence quenchers. The efficiency of fluorescence quenching should be estimated for two kinds of fluorescence dyes within the antigen-binding pocket of IgG, according to both fluorescence quenchers. Quantitative estimation of the efficiency of fluorescence quenching (E) was carried out using the calculation method of FRET efficiency, with the following equation:

$$E = 1 - (F/F_i) \quad (1)$$

where F represents the donor emission when the quencher is bound, and F_i represents the donor emission when the quencher is unbound.[19] For this calculation, the fluorescence spectrum was measured for antibodies with bound switching peptides, before and after labeling with fluorescence quenchers.[26] As shown in SI Figure S1b, the fluorescence signal was observed to be reduced at different levels, according to the combination of the fluorescence dyes at the switching peptides (FAM and TAMRA) and the fluorescence quenchers at detection antibodies (TQ2 and TQ3). As previously reported,[19] the efficiency of fluorescence quenching was estimated from the fluorescence spectrum at the wavelength of the peak fluorescence signal. As summarized in SI Table S1, E was calculated to be 0.33 for FAM at a H2-peptide and TQ2 as a fluorescence quencher at the detection antibody, 0.43 for FAM at a L2-peptide and TQ2, 0.16 for TAMRA at a L1-peptide and TQ3, and 0.28 for TAMRA at a H1-peptide and TQ3. These results showed that the L2-peptide with FAM had a higher efficiency of fluorescence quenching with TQ2 than the H2-peptide with FAM. In case of the detection antibodies with TQ3 as a fluorescence quencher, the H1-peptide with TAMRA had a higher efficiency of fluorescence quenching than L1-peptide with TAMRA.

From the efficiency of fluorescence quenching, the distance between fluorescence dyes at the binding pocket of IgG and fluorescence quenchers was estimated using the procedure described by Hink et al.[32] As the first step, the Förster distance [R_0, FRET distance (Å)] was estimated according to the following equation:

$$R_o = 0.2108 \times (\kappa^2 \times n^{-4} \times \Phi \times J)^{(1/6)} \quad (2)$$

where κ^2 is approximated to be $^2/_3$ when the donor and acceptor orientations can be freely rotated,[19,27] n represents the refractive index of the medium, Φ represents the extinction coefficients of the fluorophores, and J represents the spectral

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overlap integral between the donor and acceptor.[26] Next, the distance (R) from fluorescence dyes (donor) to the fluorescence quenchers was calculated according to the following equation:

$$R = R_0 \times [(1 - E)/E]^{(1/6)} \quad (3)$$

where E represents the efficiency of fluorescence quenching. As summarized in SI Table S1, the distance (R) from fluorescence dyes (donor) to the fluorescence quenchers was calculated to be 55.8 Å for H2-peptide labeled with FAM and TQ2, 52.6 Å for L2-peptide labeled with FAM and TQ2, 54.5 Å for L1-peptide labeled with TAMRA and TQ3, and 47.6 Å for H1-peptide labeled with TAMRA and TQ3.

Based on the distance between fluorescence dyes at the binding pocket of IgG and fluorescence quenchers, the binding sites of fluorescence quenchers at IgG were investigated using the docking model of switching peptides and IgG (PDB ID: 1HZH). Usually, IgGs are known to have about 84 potential reactive lysines that are located in the heavy chain of IgG (within the residue numbers of 279−339), as well as, the light chain of IgG (within the residue numbers of 103−169),[28] and the labeling of fluorescence quenchers is known to occur at the Fc region of antibodies. The distance between the binding pocket of IgG and lysine residues was estimated using the IgG model in Pymol software, as shown in SI Figure S1c. When the distance of these lysine residues at IgG from fluorescence dyes at the binding pocket was considered to be 56 Å, four candidate lysines were chosen to be 57.4 Å for K^{287}, 58.3 Å for K^{339}, 54.4 Å for K^{341}, and 54.1 Å for K^{345}. The fluorescence quenchers were covalently labeled to primary amines of lysines at antibodies through the N-hydroxysuccinimide ester of fluorescence quenchers.[28] In order to maximize the labeling of fluorescence quenchers to IgG, the excess amount (more than 10-fold) of fluorescence quencher was reacted to IgG of IgG, and the labeled IgG after purification was used for one-step immunoassay.

Development of the Homogeneous One-Step Immunoassay. For the homogeneous immunoassay of influenza virus, a fluorescence quencher was labeled to detect antibodies in solution (unimmobilized detection antibodies), and switching peptides were bound to the detection antibodies. As the fluorescence quenchers labeled with detection antibodies adsorbed the fluorescence from the switching peptides, no significant fluorescence signal was observed before sample treatment. Only when the influenza virus in the sample was bound to the antibodies, the switching peptides were quantitatively dissociated, as shown in Figure 1c. As previously mentioned, the switching peptides bound to specific regions within the antigen-binding pocket of IgG. Usually, each type of antigen is known to interact with specific regions of the binding pocket of IgG.[2] Hence, the specific binding of antigens could occur at different regions within the antigen-binding pocket of IgG, which also located the binding sites for four kinds of switching peptides. When a binding site of a certain switching peptide is located near the antigen-binding sites, the switching peptide is mainly dissociated from the antigen-binding pocket of IgG, according to the concentrations of bound antigens.[1] Therefore, a specific switching peptide should be selected according to the target analyte (antigen), for an effective one-step immunoassay. For this reason, a one-step immunoassay of Inf-A and Inf-B was prepared by means of (1) selection of optimal switching peptides for the detection of Inf-A and Inf-B and (2) determination of optimal concentrations of each selected switching peptide.

As the first step, the optimal switching peptide among the four types of switching peptides was selected for the detection of antibodies against Inf-A. The immobilized detection antibodies were treated with four kinds of switching peptides at the same concentration (100 nM), and then an Inf-A sample at the same concentration was reacted with this system, to dissociate the switching peptides. As shown in SI Figure S2a, the fluorescence signal was measured before and after the Inf-A sample treatment, and the relative change in fluorescence signal (%) was calculated as follows:

$$\text{relative signal}(\%) = F/F_0 \times 100 \quad (4)$$

where F and F_0 represent the fluorescence intensity after and before the sample treatment, respectively. For the two switching peptides labeled with TAMRA, the relative change in fluorescence signal was 176% for L1-peptide and 130% for H1-peptide (n = 3). For the other two switching peptides labeled with FAM, the relative change in fluorescence signal was 225% for L2-peptide and 352% for H2-peptide (n = 3). Additionally, the dissociation of the H2-peptide produced a similar fluorescence signal of 3700 AU in comparison to that of the L2-swiching peptide, and H2-peptide [1000 AU, n = 3] was estimated to display stronger binding than L2-peptide [1700 AU, n = 3], by comparing the fluorescence signal before the sample treatment. These results showed that the dissociation of switching peptides from the detection antibody against Inf-A was observed to be the highest for L1-peptide labeled with TAMRA and H2-peptide labeled with FAM, which were then considered to be optimal for the one-step immunoassay.

To determine the optimal concentration of switching peptides for the one-step immunoassay, the detection antibodies labeled with fluorescence quencher were treated with the above switching peptides for the detection of Inf-A: L1-peptide labeled with TAMRA and H2-peptide labeled with FAM. In order to determine the maximum binding of switching peptides to the detection antibodies, the switching peptides in the concentration range of 1−100 μM were used to detect antibodies labeled with fluorescence quenchers at a concentration of 250 μg/mL (L1-peptide labeled with TAMRA + detection antibodies labeled with TQ3; H2-peptide labeled with FAM + detection antibodies labeled with TQ2). As shown in SI Figure S2b, the fluorescence signal from the detection antibody was measured, and the optimal concentration of switching peptides was determined to be approximately 30 μM for the detection antibodies with both types of fluorescence quenchers (TQ2 and TQ3).

As the next step, the optimal switching peptide was selected for the detection of antibodies against Inf-B among the four types of switching peptides. The experiment was performed as described previously for Inf-A. As shown in SI Figure S2c, the fluorescence signal was measured before and after the Inf-B sample treatment, and the relative change in fluorescence signal (%) was calculated to be 180% for L1-peptide and 145% for H1-peptide (n = 3). For the other two switching peptides labeled with FAM, the relative change in fluorescence signal was 151% for L2-peptide and 199% for H2-peptide (n = 3). These results showed that the dissociation of switching peptides from the detection antibody against Inf-B was the highest for the H2-peptide labeled with FAM. Among the four switching peptides, L1-peptide labeled with TAMRA and H2-

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peptide labeled with FAM were also determined to be optimal for the one-step immunoassay of Inf-B. To determine the optimal concentration of switching peptides for the one-step immunoassay, the detection antibodies labeled with fluorescence quencher were treated with the above switching peptides, for the detection of Inf-B: L1-peptide labeled with TAMRA and H2-peptide labeled with FAM. To determine the maximum binding of switching peptides to the detection antibodies, the same experiment was carried out as described for Inf-A. As shown in SI Figure S2d, the fluorescence signal from the detection antibody was measured, and the optimal concentration of switching peptides was determined to be approximately 30 μM for the detection antibodies with both types of fluorescence quenchers (TQ2 and TQ3).

In principle, the one-step immunoassay is based on switching peptides required for the prebinding of switching peptides to the detection antibodies and the quantitative dissociation of the fluorescence-labeled switching peptides as soon as the antigen binds to the detection antibodies. Therefore, the binding affinity of target analytes (antigens) to the detection antibodies should be stronger than that of the switching peptides.[1] In this work, the binding affinities (K_D) of target analytes (Inf-A and Inf-B) and the selected switching peptides (L1- and H2-peptides) to both the detection antibodies against Inf-A and Inf-B were estimated using a SPR biosensor. As shown in SI Figure S3a, detection antibodies against the targeted influenza virus (Inf-A or Inf-B) were immobilized on the Au chip, and the unoccupied surface of the Au chip was blocked with bovine serum albumin (BSA) solution. For the analysis of binding affinities (K_D), standard samples of targeted influenza virus (Inf-A or Inf-B), and the selected switching peptides (L1- and H2-peptides) at known concentrations were treated with the immobilized detection antibodies. As shown in SI Figure S3b, the SPR signal was calculated for each sample concentration from the difference in signals between the baseline before sample injection (red arrow) and the baseline after washing (blue arrow). The SPR signal at different sample concentrations was fitted using an isotherm model for adsorption:

$$R = R_{max}\cdot([Ag])/([Ag] + K_D) \quad (5)$$

where $R(R_{max})$ represents the (maximum) response from antigen binding, [Ag] represents the concentration of antigen, and K_D represents the binding affinity.[33−37] From the reciprocal plot of the isotherm model ($1/R$ versus $1/[Ag]$), the binding affinities (K_D) were estimated to be 3.6×10^{-8} M for Inf-A, 1.4×10^{-5} M for L1-peptide, and 3.7×10^{-5} M for H2-peptide. The same experiment was carried out to detect antibodies against Inf-B. As shown in SI Figure S3c, the binding affinities (K_D) were estimated to be 2.1×10^{-8} M for Inf-B, 1.4×10^{-5} M for L1-peptide, and 5.6×10^{-5} M for H2-peptide. The binding affinities (K_D) are summarized in Table 2. These results showed that the detection antibodies against Inf-A (or Inf-B) had far stronger binding to Inf-A (or Inf-B) than the two switching peptides, and these switching peptides were dissociated from the binding pocket of IgG when Inf-A (or Inf-B) bound to the detection antibodies. From these results, the one-step immunoassays based on switching peptides for the detection of Inf-A and Inf-B were determined to be feasible using the selected switching peptides (L1- and H2-peptides).

2.3. Application of the One-Step Immunoassay for Detection of Influenza Virus. The cross-reactivity of detection antibodies against Inf-A was applied for the selective detection of Inf-A using a one-step immunoassay based on switching peptides. In case of the one-step immunoassay for detection of Inf-A, two pairs of detection antibodies against Inf-A were labeled with the fluorescence quenchers TQ2 and TQ3, while the corresponding switching peptides were labeled with the fluorescent dyes FAM and TAMRA, respectively: anti-Inf-A antibodies (TQ2) + H2-peptide (FAM) and anti-Inf-A antibodies (TQ3) + L1-peptide (TAMRA). As shown in Figure 2a, the two pairs of detection antibodies with switching peptides produced significantly higher fluorescence signals upon treatment with Inf-A, as compared to that upon treatment with Inf-B and other proteins (CRP and BSA). These results showed that these two pairs of detection antibodies with switching peptides could be used for the selective detection of Inf-A.

Table 2. Affinity Constants (K_D) of Antibodies Against Inf-A and Inf-B

	K_D value		
	Inf virus	L1-peptide	H2-peptide
anti-Inf-A antibody	3.55×10^{-8} M	1.43×10^{-5} M	3.71×10^{-5} M
anti-Inf-B antibody	2.12×10^{-8} M	1.42×10^{-5} M	5.64×10^{-5} M

The homogeneous one-step immunoassay of Inf-A was carried out using Inf-A samples in PBS. As shown in Figure 2b, the standard samples were prepared by means of serial dilution in PBS of heat-treated Inf-A [Influenza Virus, Type A (H1N1)] from Meridian Bioscience in the concentration range of 0.1−1000 ng/mL (n = 3). The fluorescence signal was observed to linearly increase in the corresponding concentration range and the limit of detection (LOD) was estimated to be 0.02 ng/mL (n = 3) for anti-Inf-A antibodies (TQ2) + H2-peptide (FAM) and 0.15 ng/mL (n = 3) for the anti-Inf-A antibodies (TQ3) + L1-peptide (TAMRA). Such a linear correlation was mainly occurred because the concentration of antibodies was far higher than the influenza antigens in the one-step immunoassay.[38,39] These results showed that both types of detection antibodies could have a similar response for the detection of Inf-A.

Real sample analysis was performed using NATtrol Influenza A Stock from Zeptometrix, which included heat-treated Inf-A in a real sample matrix. Real samples were prepared with the critical cycle number (Ct) in the range of 36.3−30, by serial dilution in a real control sample matrix (NATtrol Influenza Stock). As shown in Figure 2c, the fluorescence signal was observed to linearly increase in the corresponding concentration range, and the LOD was estimated to be Ct = 35.3 (n = 3) for anti-Inf-A antibodies (TQ2) + H2-peptide (FAM) and Ct = 35.8 (n = 3) for anti-Inf-A antibodies (TQ3) + L1-peptide (TAMRA). Such a linear correlation was also considered to be occurred because the concentration of antibodies was far higher than the influenza antigens in the one-step immunoassay. These results showed that both types of detection antibodies could have a similar response for the detection of Inf-A. As negative controls, standard samples of CoV strain 229E were prepared, and a one-step immunoassay was carried out using the same two pairs of detection antibodies with switching peptides. The assay results with the negative control were maintained at the baseline. These results showed that the homogeneous one-step immunoassay

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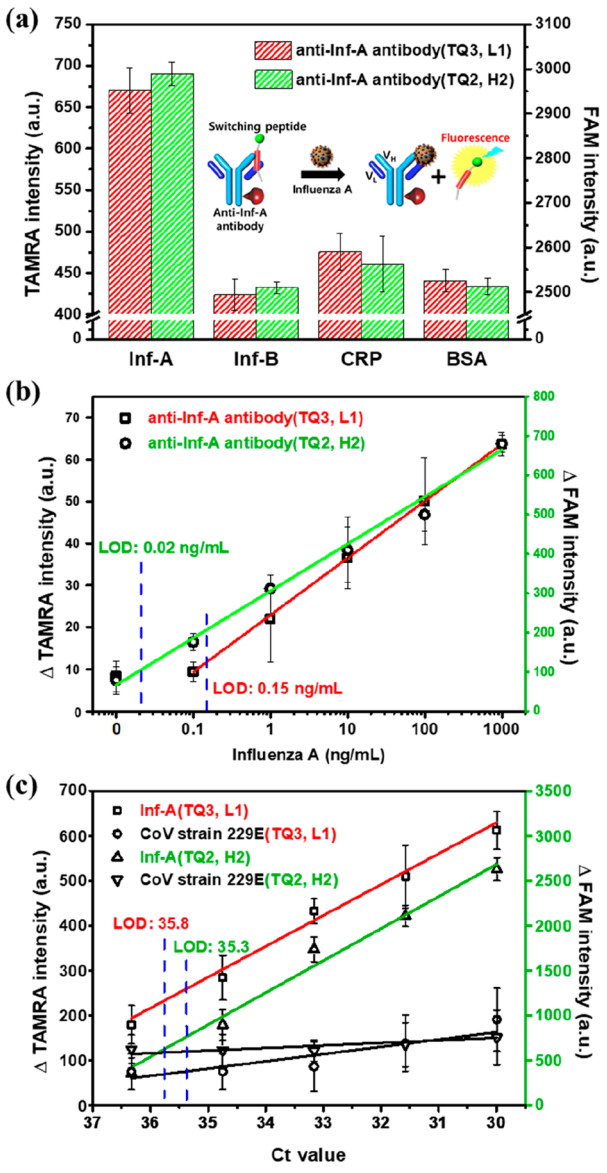

Figure 2. Homogeneous one-step immunoassay for Inf-A detection. (a) The cross-reactivity of detection antibodies against Inf-A. (b) Detection of Inf-A standard sample using homogeneous one-step immunoassay. (c) Detection of Inf-A real sample using homogeneous one-step immunoassay.

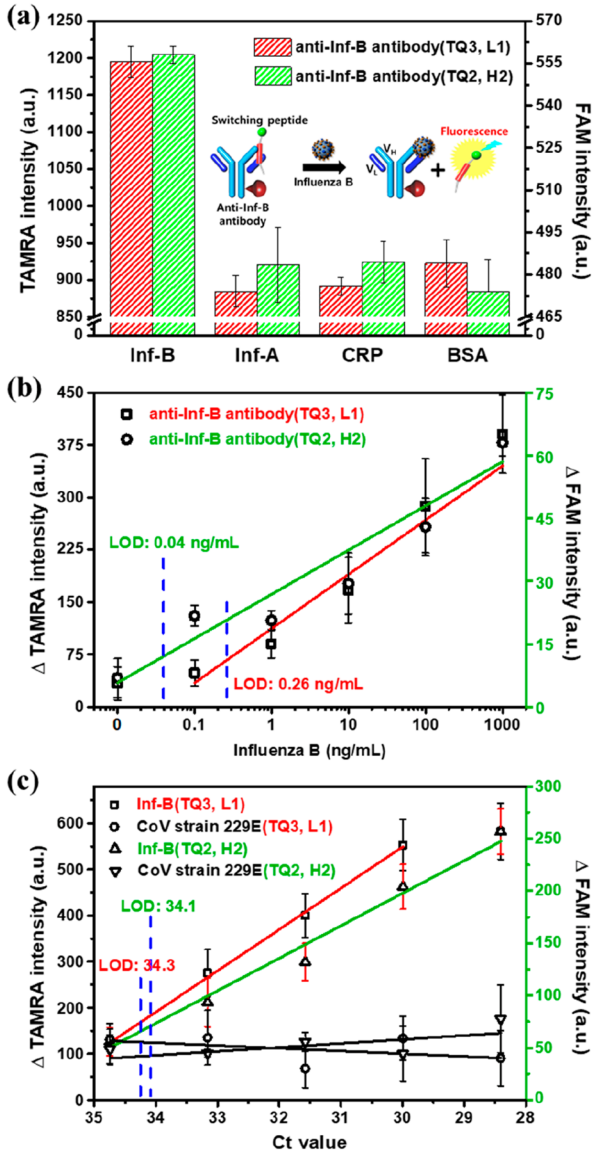

Figure 3. Homogeneous one-step immunoassay for Inf-B detection. (a) The cross-reactivity of detection antibodies against Inf-B. (b) Detection of Inf-B standard sample using homogeneous one-step immunoassay. (c) Detection of Inf-B real sample using homogeneous one-step immunoassay.

based on both types of detection antibodies could be used for the detection of Inf-A in real samples.

The cross-reactivity of detection antibodies against Inf-B was also estimated to demonstrate the selective detection of Inf-B using a one-step immunoassay based on switching peptides. The same experiment was carried out using two pairs of detection antibodies: anti-Inf-B antibodies (TQ2) + H2-peptide (FAM) and anti-Inf-B antibodies (TQ3) + L1-peptide (TAMRA). As shown in Figure 3a, two pairs of detection antibodies with switching peptides also produced significantly higher fluorescence signals upon treatment with Inf-B, in comparison to treatments with Inf-A and other proteins (CRP and BSA). These results showed that two pairs of detection antibodies with switching peptides could be used for the selective detection of Inf-B.

The homogeneous one-step immunoassay of Inf-B was carried out using Inf-B samples in PBS. As shown in Figure 3b, the standard samples were prepared by means of serial dilution in PBS of heat-treated Inf-B (Influenza B virus, Strain B, Meridian Bioscience) in the concentration range of 0.1−1000 ng/mL (n = 3). The fluorescence signal was observed to linearly increase in the corresponding concentration range and the LOD was estimated to be 0.04 ng/mL (n = 3) for anti-Inf-B antibodies (TQ2) + H2-peptide (FAM) and 0.26 ng/mL (n = 3) for anti-Inf-B antibodies (TQ3) + L1- peptide (TAMRA). These results showed that both types of detection antibodies could have a similar response for the detection of Inf-B.

Real sample analysis was performed using NATtrol Influenza B Stock from Zeptometrix, which included heat-treated Inf-B in a real sample matrix. As in case of the previous experiment for Inf-A, the real samples were prepared with Ct in the range of 34.7−28.4, by means of serial dilution in real control sample

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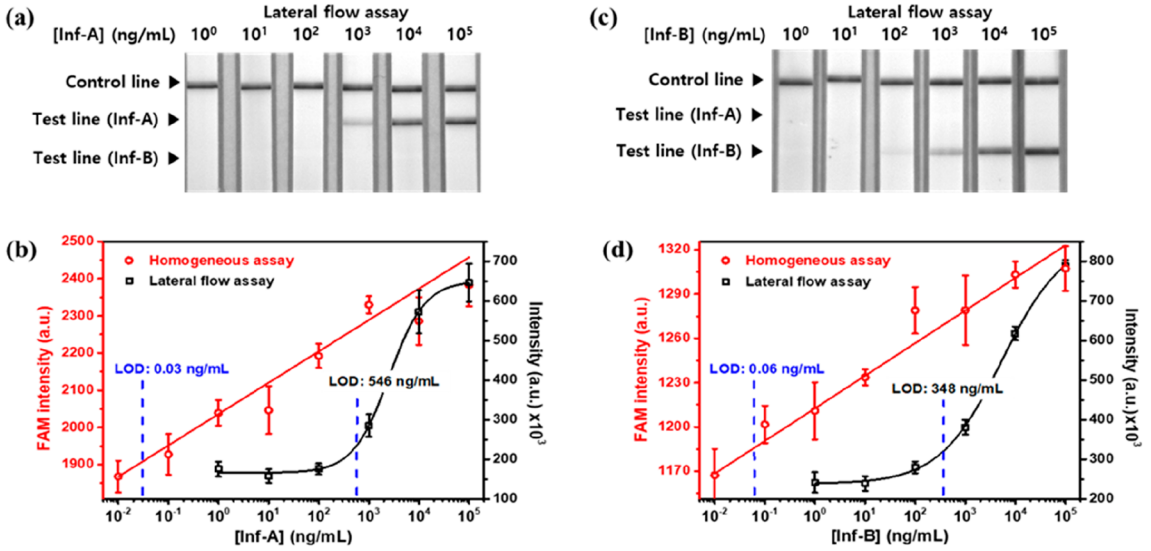

Figure 4. Comparison of commercial rapid test kits and homogeneous one-step immunoassays. (a) Immunoassay with the rapid kit for Inf-A. (b) Comparison of the two methods for Inf-A detection range and limit of detection. (c) Immunoassay with the rapid kit for Inf-B. (d) Comparison of the two methods for Inf-B detection range and limit of detection.

matrix (NATtrol Influenza Stock) from Zeptometrix. As shown in Figure 3c, the fluorescence signal was observed to linearly increase in the corresponding concentration range, and the LOD was estimated to be Ct = 34.1 (n = 3) for anti-Inf-B antibodies (TQ2) + H2-peptide (FAM) and Ct = 34.3 (n = 3) for anti-Inf-B antibodies (TQ3) + L1-peptide (TAMRA). These results showed that both types of detection antibodies could have a similar response for the detection of Inf-B. As negative controls, standard samples of CoV strain 229E were also prepared for the one-step immunoassay and the assay results with the negative control were observed to be maintained at the baseline. These results showed that the homogeneous one-step immunoassay based on both types of detection antibodies could be used for the detection of Inf-B in real samples.

The immunoassay developed in this study was compared with commercially available rapid test kits for Inf-A and Inf-B from SD Biosensor.

The influenza virus sample was prepared using inactivated influenza virus (A/New caledonia/20/99 and B/Tokio/53/99) from Meridian Bioscience, by serial dilution in sample buffer from SD Biosensor. As shown in Figure 4a, the immunoassay with the rapid kit for Inf-A was carried out in the concentration range of $1-10^5$ ng/mL. The LOD was estimated to be less than 10^5 ng/mL from the apparent test line. The standard curve for standard samples of Inf-A was compared to the results from the one-step immunoassay based on switching peptides and rapid test, as shown in Figure 4b. The detection range was estimated to be 10^3-10^5 ng/mL for the rapid test kit, with an LOD of 546 ng/mL (n = 3). In comparison to the one-step immunoassay based on switching peptides with the detection range of $0.1-10^3$ ng/mL and LOD of 0.02 ng/mL (n = 3), the one-step immunoassay based on switching peptides was feasible for a significantly sensitive detection of Inf-A in a far wider detection range than the conventional rapid test.

The immunoassay with the rapid kit for Inf-B was also carried out in the same concentration range of Inf-A ($1-10^5$ ng/mL). The LOD was estimated to be approximately 102 ng/mL from the apparent test line, as shown in Figure 4c. The standard curve for standard samples of Inf-B was compared with the results of the one-step immunoassay based on switching peptides and the rapid test, as shown in Figure 4d. The detection range was estimated to be 10^2-10^5 ng/mL for the rapid test kit, with an LOD of 348 ng/mL (n = 3). In comparison, the one-step immunoassay based on switching peptides had a detection range of $0.1-10^3$ ng/mL, with an LOD of 0.04 ng/mL (n = 3), and thus, was feasible for a significantly sensitive detection of Inf-B, in a far wider detection range than the conventional rapid test. These results showed that LOD of the one-step immunoassay was far lower than the conventional lateral-flow immunoassay and the dynamic range of the one-step immunoassay could cover the undetectably low concentration range of the lateral-flow immunoassay.

CONCLUSIONS

In the present study, a homogeneous one-step immunoassay based on switching peptides has been presented for the detection of Inf-A and -B. The one-step immunoassay involves just treatment of the sample, without any washing steps. In this method, fluorescence-labeled switching peptides were quantitatively dissociated from the antigen-binding site of IgG. In particular, the one-step immunoassay based on soluble detection antibodies with switching peptides is called a homogeneous one-step immunoassay. The homogeneous one-step immunoassay was developed using switching peptides labeled with two types of fluorescence dyes (FAM and TAMRA) and detection antibodies labeled with two types of fluorescence quenchers (TQ2 for FAM and TQ3 for TAMRA). The optimal switching peptides for the detection of Inf-A and Inf-B were selected as L1-peptide and H2-peptide. The interactions between the four kinds of switching peptides and IgG have been analyzed using computational docking simulation and SPR biosensor. The location of labeling for the fluorescence quenchers was analyzed from the distance between the fluorescence dyes of the switching peptides and the fluorescence quenchers, which were calculated from the efficiency of fluorescence quenching, using the Förster

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equation. To demonstrate the feasibility of the one-step immunoassay, the binding constants (K_D) were calculated for Inf-A and Inf-B detection antibodies against target antigens (Inf-A and Inf-B) and switching peptides (L1- and H2-peptides), using an isotherm model. The homogeneous one-step immunoassay of Inf-A and Inf-B was demonstrated to be feasible using antigens, as well as, real samples of Inf-A and Inf-B, with a critical cycle number (Ct). The one-step immunoassays of Inf-A and Inf-B based on switching peptides were compared with commercially available for test kits for Inf-A and Inf-B, and found to be far more sensitive in comparison to the conventional rapid tests, over the entire detection range.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.2c00716.

(PDF)

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Notes

The authors declare no competing financial interest.

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