Article

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A multispecific monoclonal antibody G2 recognizes at least three completely different epitope sequences with high affinity

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ABSTRACT

A monoclonal antibody (mAb) G2 possesses an unusual characteristic of reacting with at least three proteins (ATP6V1C1, SEPT3, and C6H10orf76) other than its original antigen, chicken prion protein (ChPrP). The epitopes on ChPrP and ATP6V1C1 have been identified previously. In this study, we identified the epitope in the third protein, SEPT3. Interestingly, there was no amino acid sequence similarity among the epitopes on the three proteins. These epitopes had high binding affinities to G2 (K_D = ~10^{-7} M for monovalent binding and K_D = ~10^{-9} M for divalent binding), as determined using a SPR biosensor. This is the first report on a three-in-one mAb recognizing completely different epitope sequences with high affinity. Additionally, competitive ELISA indicated that the binding sites on G2, specific for the three different epitopes, overlapped, suggesting that the antigen-binding site may be flexible in the free form and capable of adapting to at least three different conformations to enable interactions with three different antigens.

Keywords: multispecific antibody; epitope mapping; antigen-recognition mechanism; surface plasmon resonance

Abbreviations: Ag, antigen; ATP6V1C1, V-type proton ATPase subunit C 1; C6H10orf76, chicken chromosome 6 C10orf76 homolog; ChPrP, chicken PrP; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunosorbent assay; Fab, antigen-binding fragment; GST, Glutathione S-transferase; HER3, human epidermal growth factor receptor 3; HRP, horseradish peroxidase; IPTG, isopropyl β-D-1-thiogalactopyranoside; ITC, isothermal titration calorimetry; K_D, equilibrium dissociation constant; k_{off}, dissociation rate constant; k_{on}, association rate constant; mAb, monoclonal antibody; PBST, PBS containing 0.1% Tween 20; PrP, prion protein; PVDF, polyvinylidene fluoride; RU, response unit; RU_{eq}, RU at equilibrium; scFv, single-chain variable fragment; SEPT3, Neuronal-specific septin-3; SPR, surface plasmon resonance

Introduction

In general, monoclonal antibody (mAb)-antigen (Ag) interactions are extremely specific and a mAb binds only one Ag.[1] This highly specific one-to-one recognition is one of the primary reasons for the success of antibodies as biological tools and targeted therapeutics.[2,3] However, a few mAbs can bind more than one Ag specifically, such as G2 described in this paper.[4] It is suggested that this multispecificity helps increase the diversity of mAb repertoire,[5] confers advantages to pathogen-specific antibodies,[6,7] and has advantages in therapeutic applications.[8,9] One of the therapeutic applications is the two-in-one multispecific mAb, MEHD7945A, against human epidermal growth factor receptor 3 (HER3) and epidermal growth factor receptor (EGFR) for cancer treatment.[8] This mAb blocks two parallel cancer signaling pathways and is broadly efficacious in multiple tumor models compared with mono-specific anti-HER antibodies. Therefore, multispecific mAbs may provide new opportunities for antibody-based therapies. However, reports concerning multispecific mAbs are still limited, and the detailed mechanism associated with multispecificity remains unknown.

G2 was generated by immunizing mice with recombinant chicken prion protein (ChPrP) residues 174–247;[10] however, G2 reacted with at least three proteins (ATP6V1C1, SEPT3, and C6H10orf76) other than the original Ag.[4] The epitope sequences in ChPrP and the second Ag protein, ATP6V1C1, have been identified previously.[4] Interestingly, there was no amino acid sequence homology among ChPrP, ATP6V1C1, SEPT3, and C6H10orf76. Therefore, the third epitope on SEPT3 for G2 may be different compared with those on ChPrP and ATP6V1C1. To the best of our knowledge, there are no reports on such three-in-one type mAbs capable of recognizing completely different epitope sequences with high affinity. In this study, we identified the third epitope recognized by G2 in SEPT3 protein and proposed a binding model for the multispecific mAb G2.

Results

Identification of the amino acid sequence of the third epitope on SEPT3 recognized by G2

In our previous study,[4] we immunoscreened a complementary DNA library from chicken brain, found three proteins SEPT3, ATP6V1C1 and C6H10orf76 which reacted with G2, and identified the epitope sequence of ATP6V1C1 among these. In this study, we analyzed the epitope sequence of SEPT3 on the clone coding SEPT3, F1. From the result of DNA sequencing, F1 had a sequence encoding the N-terminal region-truncated SEPT3. Here, to identify the amino acid sequence specific for G2 binding, several SEPT3 DNA fragments of different sizes were amplified using various primers (Table S1) and inserted into the expression vector pRSET-B to express the peptides in *Escherichia coli* BL21 cells [Fig. 1(A)]. The binding ability of G2 to the expressed peptides was assessed by western blot as described previously.[4] Figure 1(B) shows the results of the western blot experiment. G2 reacted with the B11H6 fragment, but not with the B11H5 fragment [Fig. 1(B), *lanes 5 and 7*], indicating that G2 recognized amino acid sequences between H5 and H6 sites.

To identify the more precise binding site, we synthesized three peptides (Pep37, Pep38, and Pep39) [Fig. 1(A)] and performed enzyme-linked immunosorbent assay (ELISA) and found that only Pep39 binds to G2 [Fig. 1(C)]. Further, we evaluated the contributions of the terminal amino acid of Pep39 to G2 binding. Extension of the C-terminal region of Pep39 resulted in no changes in affinity (Fig. S1), whereas extension of the N-terminal region increased G2-binding affinity [Fig. 1(C)]. The highest O.D. value of Pep395 indicated the highest affinity of Pep395 among the Pep39-derived peptides.

Comparison of G2-binding affinity to the three epitope peptides

We identified Pep395 as the third G2-specific epitope in SEPT3. The information on the three epitope peptides recognized by the multispecific mAb G2 is summarized in Table 1. To compare the binding affinity of the three epitope peptides to G2, we performed surface

plasmon resonance (SPR) experiments (Fig. 2). The Glutathione S-transferase (GST)-tagged epitope peptides[11] were immobilized on the sensor chip followed by injection of various concentrations of G2. The K_D values obtained at low mAb concentration range of 12.5–100 nM were 1.1×10^{-9} M, 9.4×10^{-9} M, and 2.9×10^{-9} M for Pep18mer, Pep8, and Pep395, respectively (Table 2, Fig. S2). Comparison of the K_D values associated with G2 binding to the three peptides showed that Pep395 could bind G2 with affinity comparable with that of Pep18mer and Pep8.

The K_D values obtained at low mAb concentration range of 12.5–100 nM revealed avidity. Because mAb has two combining sites, monovalent as well as divalent binding was observed on the Ag-immobilized sensor chip.[12,13] As shown in Figure 2(D), Scatchard plots describing binding to the immobilized Ag peptides indicated formation of two types of complexes: a divalent complex formed from two antigen-binding fragment (Fab) arms at low mAb concentrations and a monovalent complex using one Fab arm at high mAb concentrations. Therefore, the avidity can be minimized at high mAb concentrations. In the case of binding to Pep395, linear fitting of the plots in mAb concentration ranges of 1.6–6.4 μM and 12.5–100 nM provided the apparent K_D values of 3.5×10^{-7} M and 7.7×10^{-9} M, respectively. This K_D value at the mAb concentration range of 12.5–100 nM, obtained from the linear fitting of the Scatchard plot, is comparable with that obtained from curve fitting of the sensorgram using a 1:1 binding model at the same concentration range (Table 2). Similarly, based on the linear fitting to the plots in the high mAb concentrations range of 1.6–6.4 μM, the apparent K_D values for the Pep18mer and Pep8 peptides were determined to be 2.0×10^{-7} M and 6.7×10^{-7} M, respectively. Comparison of the K_D values of the three peptides with G2 under conditions minimizing avidity indicated that Pep395 could also bind G2 with comparable affinity as that shown with Pep18mer and Pep8.

Competitive ELISA

To examine the overlap of the Ag-binding sites on G2 for the three epitope peptides, we performed competitive ELISA.[14,15] Pre-incubated G2 with one of the epitope peptide was used. Pep38, which does not bind to G2 [Fig. 1(A)], was used as a negative control. Pre-incubation with Pep38 did not reduce G2-binding affinity to Pep18mer, Pep8, or Pep39 (Fig. 3, *white bars*). By contrast, pre-incubation with Pep18mer reduced the G2-binding affinity to Pep18mer, Pep8, and Pep39 (Fig. 3, *black bars*). This was consistent with our previous competitive experiment using western blot, which indicated that pre-incubation of G2 with Pep18mer prevented G2 binding to ChPrP (its epitope corresponds to Pep18mer) and ATP6V1C1 (its epitope corresponds to Pep8).[4] Pre-incubation with Pep8 or Pep39 also reduced the G2-binding affinity to Pep18mer, Pep8, and Pep39 (Fig. 3, *striped and dotted bars, respectively*). This result indicated that the binding sites of the three different epitope peptides overlapped each other.

Discussion

The third epitope peptide Pep395 binds G2 with affinity comparable with that of Pep18mer and Pep8

To evaluate the binding affinity for monovalent Ag binding and avidity for divalent Ag binding of G2 to the three epitope peptides, SPR experiments were performed, in which the epitope peptides with GST-tag were immobilized on the sensor chip and various concentrations of G2 were injected. Using this system, we can determine the K_D values at low and high mAb concentration ranges, which are mainly due to divalent and monovalent Ag binding, respectively. The K_D values of Pep18mer, Pep8, Pep395 at high G2 concentration range were 2.0×10^{-7} M, 6.7×10^{-7} M, and 3.5×10^{-7} M, respectively (Table 2). These values for Pep18mer and Pep8 were similar to those obtained by isothermal titration calorimetry (ITC) experiment previously,[4] which does not contain avidity. The K_D value for Pep395 was

also similar to that obtained for single-chain variable fragment (scFv) of G2 and Pep395 by ITC experiments (data not shown). Therefore, the K_D values for the monovalent binding presented in this study are reliable. The K_D values of Pep18mer, Pep8, and Pep395 at low G2 concentration range were 1.1×10^{-9} M, 9.4×10^{-9} M, and 2.9×10^{-9} M, respectively (Table 2). All three epitope peptides were found to strongly bind G2. We also found that all the K_D values with avidity were approximately 100-times smaller than those without avidity due to increased k_{on} and decreased k_{off} values. Interestingly, although the K_D values of the three peptides were similar, the kinetics of the binding was different (Fig. 2 and Table 2). Pep395 had larger association and dissociation rate constants (k_{on} and k_{off}) than those of Pep18mer and Pep8. This may indicate that G2 adopted different binding conformations through some different binding pathways.

G2 is a unique three-in-one type mAb that recognizes completely different epitopes with high affinity

In general, a mAb bind only one Ag[1], especially for mAb with high affinity. The divalent affinity of polyreactive mAbs for their different ligands is generally lower ($K_D = 10^{-3}$–10^{-7} M) than that of monoreactive mAbs for their cognate ligands ($K_D = 10^{-7}$–10^{-11} M).[6,16-18] For examples, divalent affinity of polyreactive rheumatoid factor mAbs were ~10^{-5} M[16] and divalent affinities of polyreactive binding of anti-HIV antibodies were 10^{-5}–10^{-6} M.[17] Compare to such polyreactive mAbs, G2 has high affinity (K_D = ~10^{-9} M for divalent binding). However, reports on multispecific mAbs with high affinity remain limited. We summarized the multispecific mAbs reported so far in Table 3. Most of them recognized homologous Ags. Moreover, except CB4-1, the multispecific mAbs recognize only two different Ags. Although, CB4-1 recognizes three different peptides, its affinities for the second and third peptides are 40-fold and 1,000-fold weaker than that for the first peptide, respectively. By contrast, G2 recognized completely different epitope sequences, Pep18mer,

Pep8, and Pep395 (Table 1) with similar K_D values, 1.1, 9.4, and 2.9 nM, respectively (Table 3). Therefore, this is the first report on a three-in-one type mAb that recognizes completely different epitope sequences with high affinity. G2 is a unique and useful target for understanding mechanisms associated with mAb-Ag recognition and mAb multispecificity.

Mechanism of the multispecificity of G2

It is known that protein structures are inherently dynamic, allowing them to transiently populate low-lying excited states or sparsely populated higher-energy states, which are characterized by short lifetimes and higher free energies relative to the predominant ground state.[19-22] The low-lying excited states sometimes exhibit different binding affinities for a substrate and acquire new functions because of the different structures between the ground and the low-lying excited states.[23] G2 multispecificity may be explained by such conformational flexibility of protein. Competitive ELISA indicated that the binding sites on G2 of the three different epitope peptides overlapped each other. Therefore, the Ag-binding site on G2 may have flexible conformation in the free form and adapt to at least three different conformations to bind three different Ags. One possible model for the multispecific recognition exhibited by G2 is described in Figure 4. Three different conformers, N_1, N_2, and N_3, exist in the free form, with these conformers undergoing rapid exchanges. The conformer N_1 may bind to the original Ag, Pep18mer, and conformers N_2 and N_3 may bind Pep8 and Pep395, respectively. Similar K_D values for the three Ags indicate that the complexes N_1+Pep18mer, N_2+Pep8, and N_3+Pep395 exhibit similar free-energy levels (Fig. 4). Interestingly, we recently succeeded to change the specificity of G2.[11] A Pro containing mutant of G2 scFv at residue 95 of the light chain lost its binding ability to Pep18mer, but remained those to Pep8 and Pep395; a single mutation can change the free-energy surface of G2. Understanding the mechanism of multispecific recognition of G2 could extend our

knowledge on the mAb-Ag recognition and help develop multispecific mAbs for therapeutic applications.

Materials and Methods

G2 expression and purification

Hybridoma producing murine mAb G2 (isotype G1) was cultured in Hybridoma-SFM (Gibco, Grand Island, NY) supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin at 37°C in a humidified 5% CO_2 atmosphere.[4,10] The expressed G2 was purified using protein A sepharose.

Construction of recombinant expression plasmids

To construct recombinant expression plasmids based on pRSET-B (Invitrogen), target genes were amplified by polymerase chain reaction using a forward primer with a *BamH*I site and a reverse primer with a *Hind*III site. Each recombinant plasmid construct was transformed into BL21 cells, and protein expression was inducted in the presence or absence of isopropyl β-D-1-thiogalactopyranoside (IPTG).[4]

Western blot analysis

Protein samples were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane for reaction with G2.[10]

Peptide synthesis and ELISA

Synthesized peptides were obtained from Sigma Life Science (Sapporo, Japan) or Scrum, Inc. (Tokyo, Japan). ELISA was used to measure *in vitro* reactions between synthetic peptides and G2 as described previously.[10] For normal ELISA, 96-well plates (Nunc, Roskilde, Denmark)

were coated with 100 ng/well of Ag peptides in 50 μl PBS (pH 7.0). After adsorption, the wells were blocked with 5% skim milk in PBS containing 0.1% Tween 20 (PBST) and then incubated with G2 at various concentrations for 1 h at 37°C. After washing with PBST, the wells were incubated with 60 μl of 1:2000-diluted horseradish peroxidase (HRP)-conjugated $F(ab')_2$ fragment of anti-mouse immunoglobulin G (GE Healthcare UK LTD, Buckinghamshire, UK). The antigen-antibody complexes were detected by adding a 100 μl substrate solution of 2,2'-azino-bis(3-ethyl-benzothiazoline-6-sulfonic acid) diammonium salt and 0.04% H_2O_2 in 50 mM citrate-phosphate buffer (pH 4.0), and the absorbance at 405 nm was measured using a microplate reader (Multiskan MS-UV, LabSystems, Uppsala, Sweden). For competitive ELISA, G2 was pre-incubated to block the binding sites with one of the Ag peptides in PBS for 5.5 h at room temperature and then used as the primary Ab in the normal ELISA experiment.

SPR measurement

Real-time mAb-Ag interactions were measured using the Biacore biosensor system (Biacore T200; GE Healthcare UK LTD, Buckinghamshire, UK). GST-tagged Ag peptides[11] (Pep18mer, Pep8, and Pep395) were immobilized on the dextran surface of a CM5 sensor chip (GE Healthcare) with amine coupling. The resultant immobilization of Pep18mer, Pep8, and Pep395 were 280, 110, and 140 RUs, respectively. G2 at various concentrations in PBS containing 0.005% Tween-20 (running buffer) were injected at a rate of 20 μL/min for 180 s. Complex dissociation was measured by injecting the running buffer alone for 180 s, followed by injecting 3 M Gdn-HCl containing 1 M acetate for 45 s as a regeneration step. Experiments were performed at 25°C, and collected data were analyzed using BIAevaluation 3.1 software (GE Healthcare). The obtained curves were fitted to a 1:1 binding model to determine the kinetic rate constants (k_{on} and k_{off}). The K_D values were calculated from the two rate constants, k_{off}/k_{on}. The K_D values were also determined by Scatchard analysis using Eq. (1):

$$RU_{eq}/c = RU_{max}/K_D - RU_{eq}/K_D \quad (1)$$

where c is the free analyte concentration, RU_{eq} is the RU at equilibrium, and RU_{max} is the total surface binding capacity.

Conflicts of interest

The authors have no conflicts of interest directly relevant to the content of this article.

Acknowledgments

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Figure legends

Figure 1. Identification of the third epitope in SEPT3 recognized by the multispecific mAb G2. (A) Schematic illustration of the peptides (gray bar) and primers (black arrow) used for identification of the SEPT3 epitope. Amino acid numbers of each peptide are shown at both ends of the bars and primer names are shown under the arrows. (B) Detection of G2 binding to the peptides by western blot analysis. Recombinant proteins expressed in *E. coli* BL21 cells were examined for interactions with G2. Lane 1, molecular sizes marker, molecular sizes are indicated on the left; lane 2, B11H3 without IPTG induction; lane 3, B11H3 with IPTG induction; lane 4, B11H5 without IPTG induction; lane 5, B11H5 with IPTG induction; lane 6, B11H6 without IPTG induction; lane 7, B11H6 with IPTG induction; lane 8, B11H7 without IPTG induction; lane 9, B11H7 with IPTG induction. (C) Comparison of G2-binding affinity

to SEPT3-derived peptides [Pep38 (triangle), Pep394 (reverse triangle), Pep39 (cross), and Pep395 (circle)] using ELISA.

Figure 2. SPR measurements for interactions between G2 and epitope peptides. SPR sensorgram for G2 binding to (A) Pep18mer, (B) Pep8, or (C) Pep395 at pH 7.4 and 25ºC. G2 concentrations were 25, 50, 100, 200, 400, 800, and 1600 nM (from bottom to top). (D) Scatchard plot describing G2 binding to each of the epitope peptides. The RU_{eq}/c values were plotted against the RU_{eq} values, where RU at equilibrium (RU_{eq}) was observed in the antibody concentration range for G2 binding to Pep18mer (○, 400 nM – 6.4 μM), Pep8 (Δ, 400 nM – 6.4 μM), and Pep395 (□, 6.25 nM – 6.4 μM) immobilized on a CM5 sensor chip.

Figure 3. Competitive ELISA experiment with the epitope peptides. G2 binding to the target peptide (%) is shown on the Y-axis and the names of the target peptides is shown on the X-axis. Unblocked G2 binding to target peptides was assumed to be 100% (*gray bar*). Binding of pre-incubated G2 with Pep38, which does not bind to G2, is a negative control (*white bar*). Binding of G2 pre-incubated with Pep18mer, Pep8, and Pep39 are represented by black, striped, and dotted bars, respectively. Error bars indicate the standard deviation obtained from three experiments.

Figure 4. A schematic view of the energy landscape of a (A) normal single-specific mAb and (B) multispecific mAb G2. The G2 ligand-unbound (*gray*) conformation is flexible and capable of exchanging between several conformations, including N_1, N_2, and N_3. For Pep18mer binding, protein can reside in one of two energy landscapes: a ligand-unbound (*gray*) or Pep18mer-bound (*black*) landscape. For Pep8 binding, protein can reside in one of two energy landscapes: a ligand-unbound (*gray*) or Pep8-bound (*broken*) landscape. For

Pep395 binding, protein can reside in one of two energy landscapes: a ligand-unbound (*gray*) or Pep395-bound (*dotted*) landscape.

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Table 1. Epitope name, original protein name, and the sequence recognized by G2.

Epitope name	Original protein name	Amino acid sequence of the epitope	Reference
Pep18mer	ChPrP	EAVAAANQTEVEMENKVV	Kamatari et al., 2014[4]
Pep8	ATP6V1C1	CQQTWEKLHAATTKN	Kamatari et al., 2014[4]
Pep395	SEPT3	LEDKTENDKIRRESMPFAVVGSDKEYQVN	This study

Table 2. Monovalent and divalent binding affinity of G2 to immobilized antigens with GST-tag.

	K_D (M)[a]	k_{on} ($M^{-1}s^{-1}$)[b,c]	k_{off} (s^{-1})[b,c]	K_D (M)[b,d]
Pep18mer	2.0×10^{-7}	$1.7 (\pm 0.4) \times 10^{5}$	$1.8 (\pm 0.7) \times 10^{-4}$	1.1×10^{-9}
Pep8	6.7×10^{-7}	$1.1 (\pm 0.3) \times 10^{5}$	$1.1 (\pm 0.2) \times 10^{-3}$	9.4×10^{-9}
Pep395	3.5×10^{-7}	$2.0 (\pm 0.5) \times 10^{6}$	$5.9 (\pm 0.0) \times 10^{-3}$	2.9×10^{-9}

a. Monovalent binding affinity of G2 obtained at higher G2 concentration ranges (1.6–6.4 μM).

b. Divalent binding affinity of G2 obtained at lower G2 concentration ranges (12.5–100 nM)

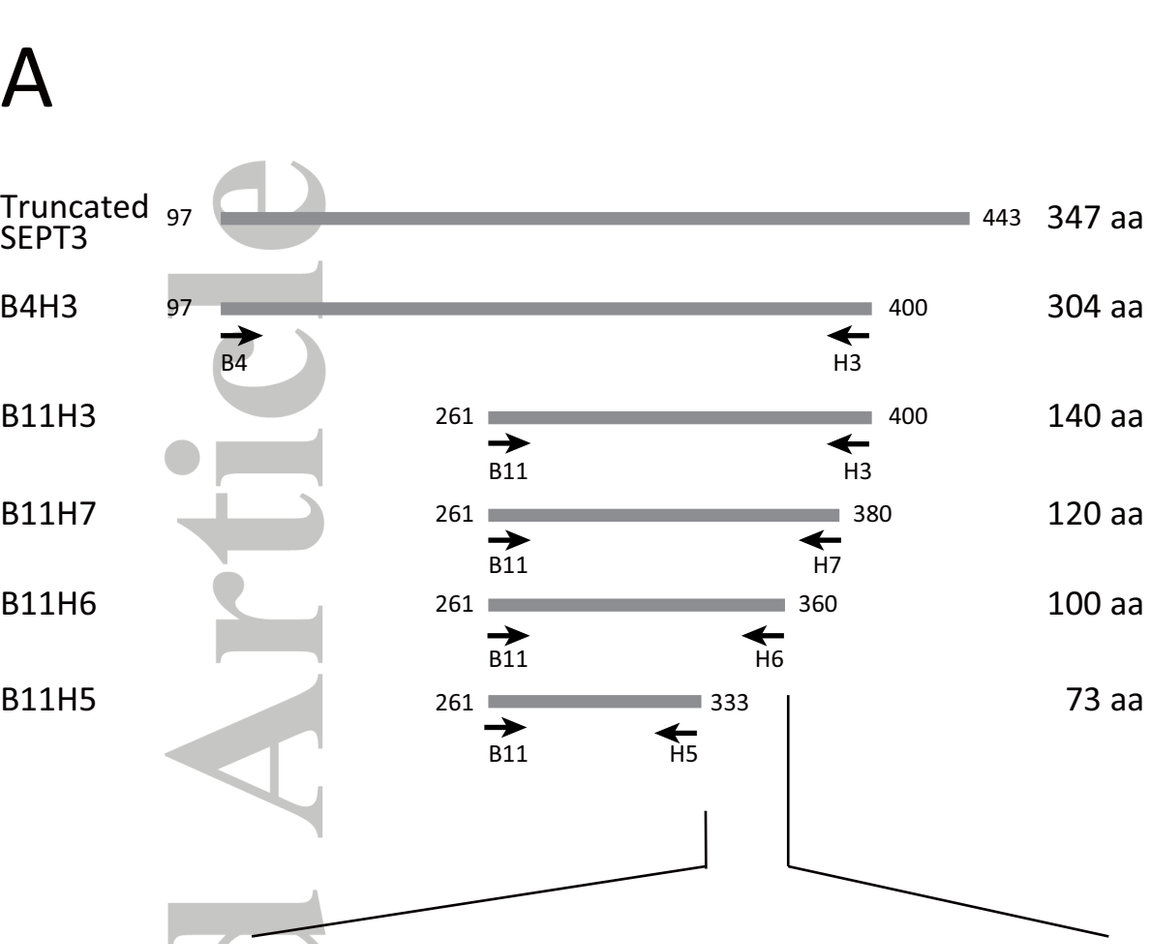
c. The k_{on} and k_{off} are the average of two experiments.

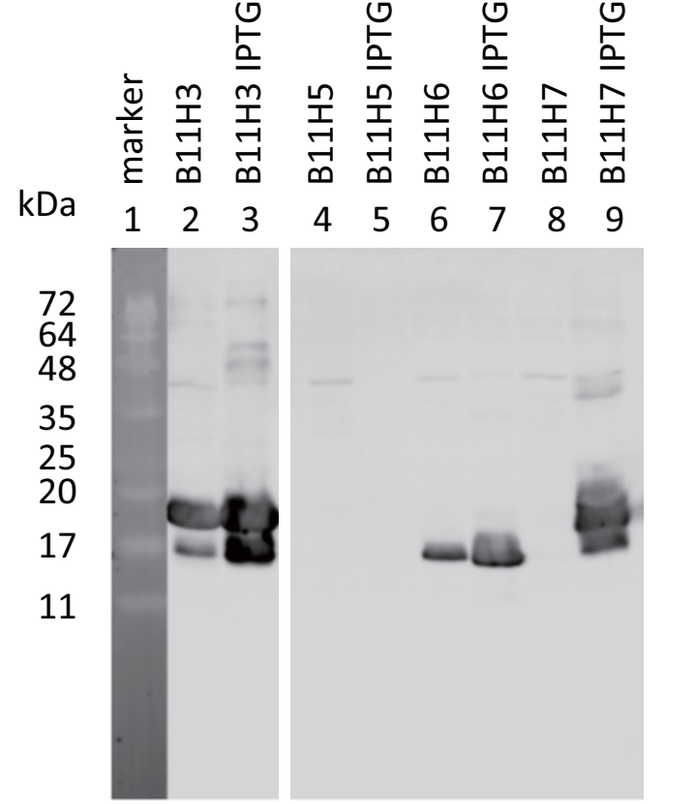
d. The K_D are calculated from the two rate constants: $K_D = k_{off}/k_{on}$.

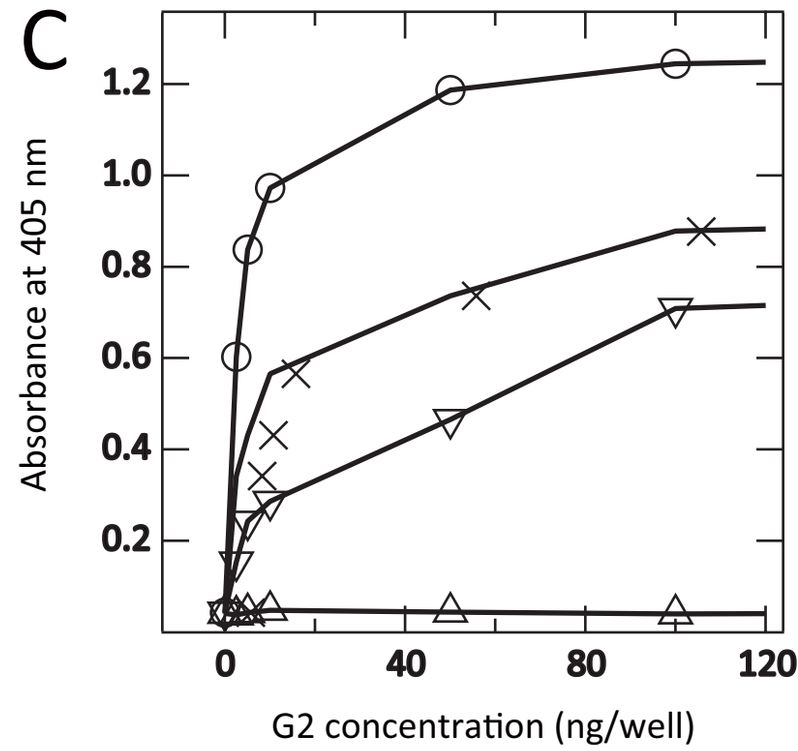
Table 3. Multispecific mAbs reported so far.

mAb name	Ag name	K_D (nM) (monovalent)	K_D (nM) (divalent)	Reference
G2	ChPrP	200	1.07	This study
	ATP6V1C1	670	9.35	This study
	SEPT3	350	2.92	This study
bH1	HER2	300	n.d.	Bostrom et al., 2009[24]
	VEGF	20	n.d.	Bostrom et al., 2009[24]
bH1-44	HER2	0.2	n.d.	Bostrom et al., 2011[9]
	VEGF	3.3	n.d.	Bostrom et al., 2011[9]
DL11f	EGFR	1.9	n.d.	Schaefer et al., 2011[8]
	HER3	0.4	n.d.	Schaefer et al., 2011[8]
5A12	VEGF	5	n.d.	Koenig et al., 2015[25]
	Ang2	5	n.d.	Koenig et al., 2015[25]
G6	mouse VEGF	0.9	n.d.	Fuh et al., 2006[26]
	human VEGF	0.9	n.d.	Fuh et al., 2006[26]
1C36	IL5	24	n.d.	Lee et al., 2014[27]
	IL4	3.5	n.d.	Lee et al., 2014[27]
CB4-1	Peptide: GATPEDLNQKLAGN	1.4	n.d.	Kramer et al., 1997[28]
	Peptide: GLYEWGGARITNTD	61	n.d.	Kramer et al., 1997[28]
	Peptide: RDFDKEWNLIEQNS	1900	n.d.	Kramer et al., 1997[28]
CB3s	mouse BR3	1.0	n.d.	Lee et al., 2006[29]
	human BR3	0.6	n.d.	Lee et al., 2006[29]
HH8	hen lysozyme	n.d.	1.1	Lavoie et al., 1999[30]
	Japanese quail lysozyme	n.d.	7.8	Lavoie et al., 1999[30]
CR2	BoNT subtype A1	0.11	0.010	Garcia-Rodriguez et al., 2007[31]
	BoNT subtype A2	0.087	0.29	Garcia-Rodriguez et al., 2007[31]
SPE7	DNP	20	n.d.	James et al., 2003[32]
	NP	100,000	n.d.	James et al., 2003[32]

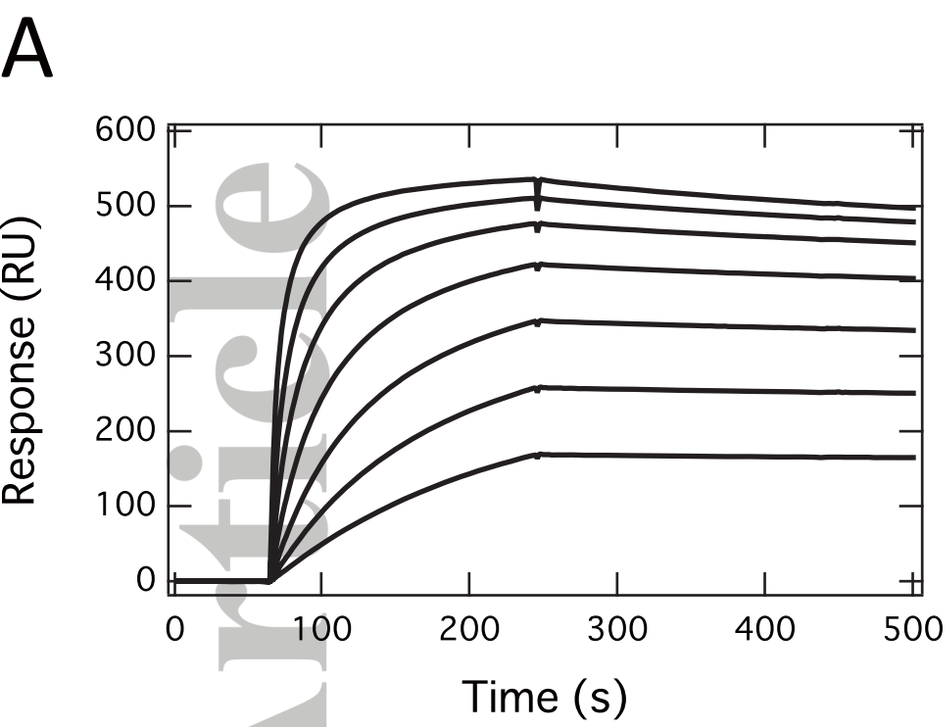
n.d., not determined.

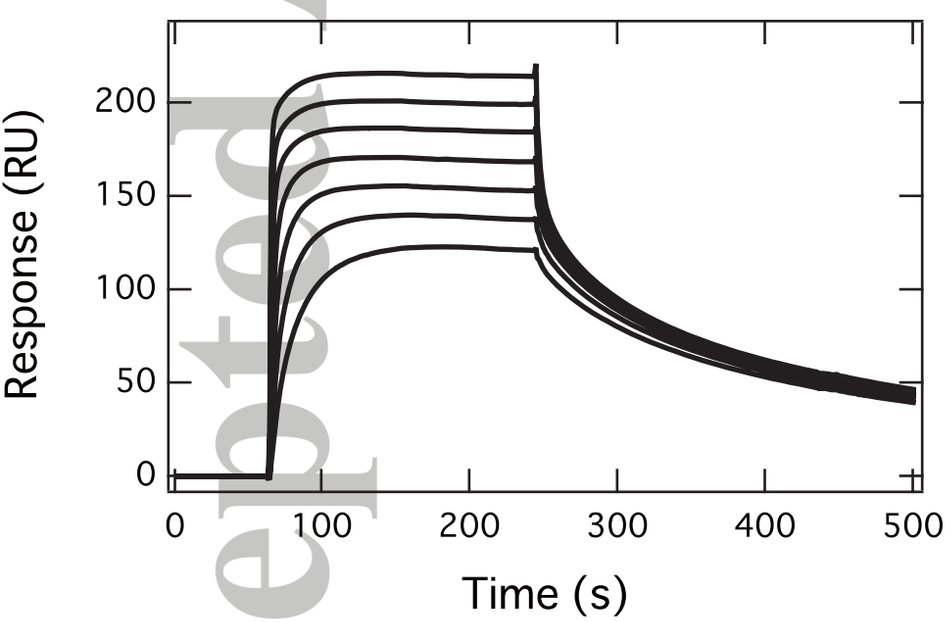


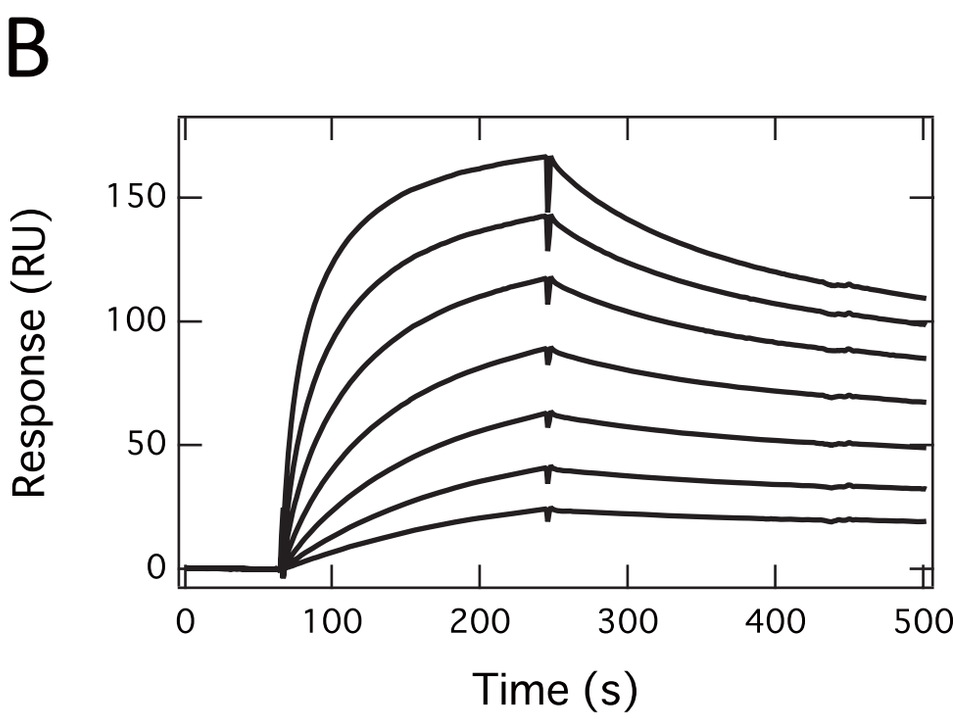


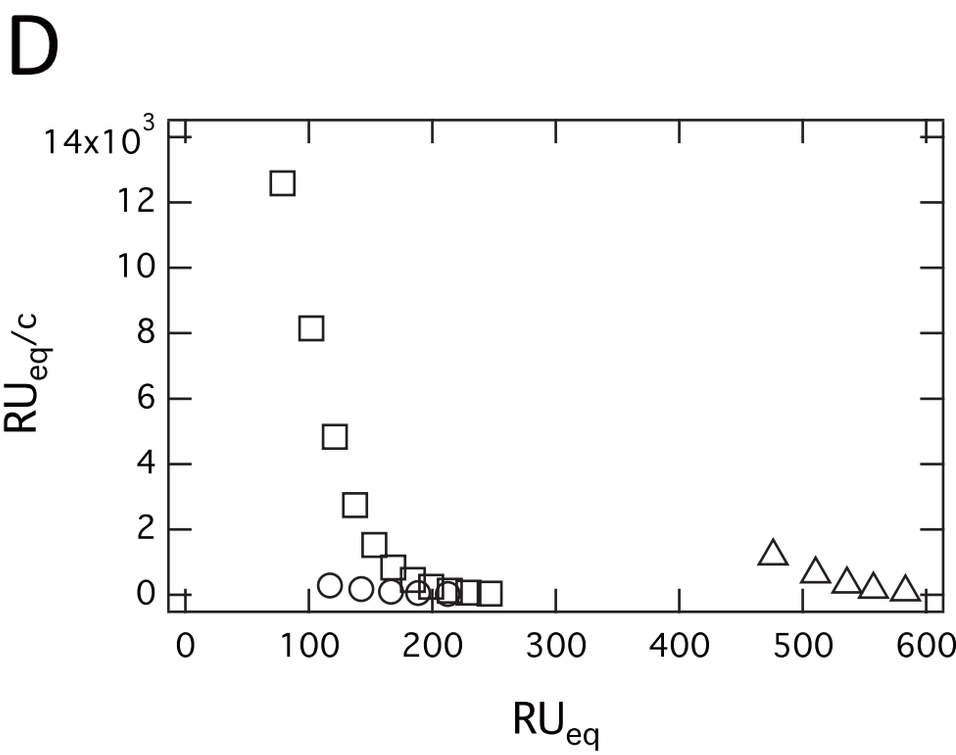


Mahmud et al., Figure 1

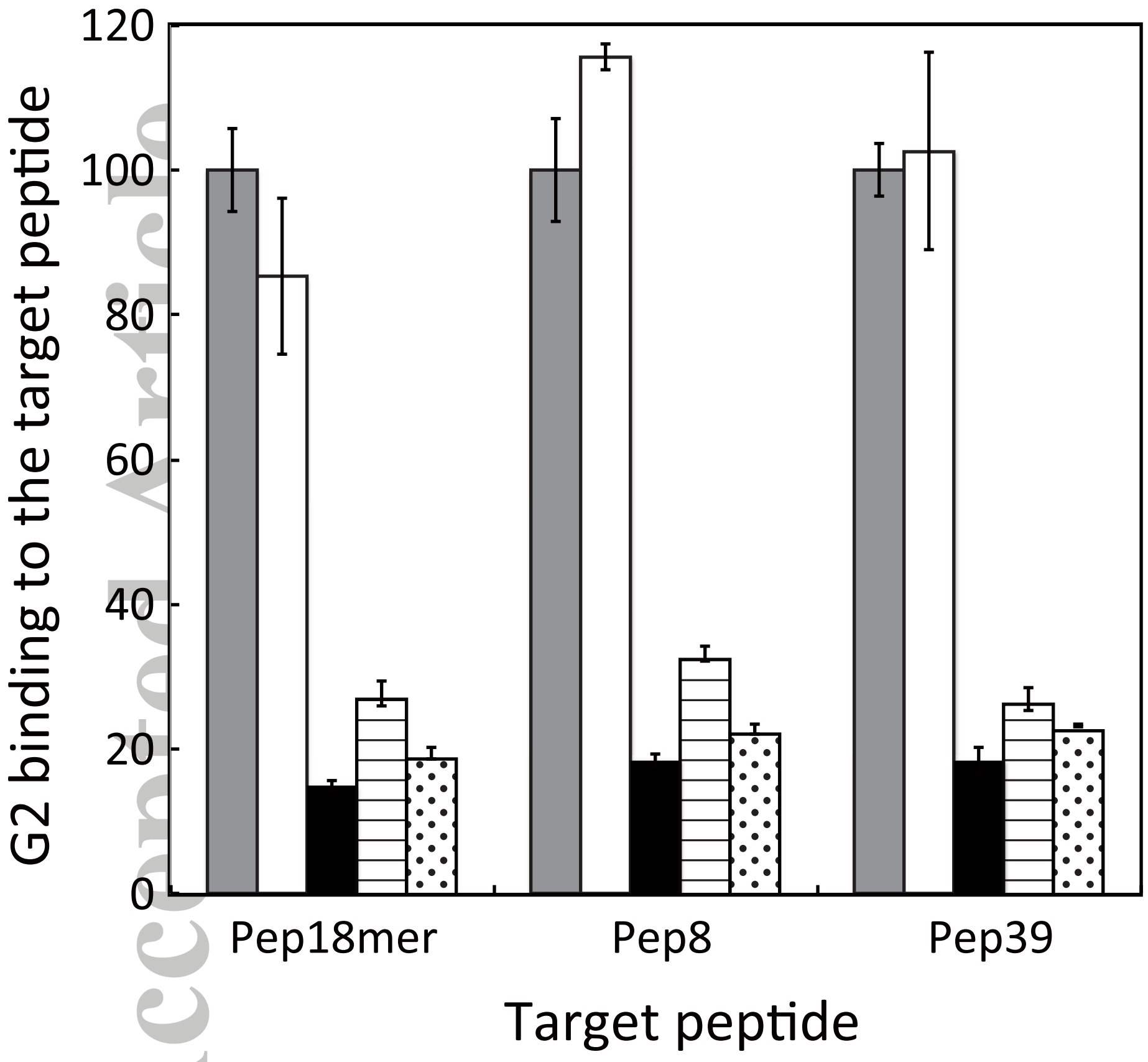




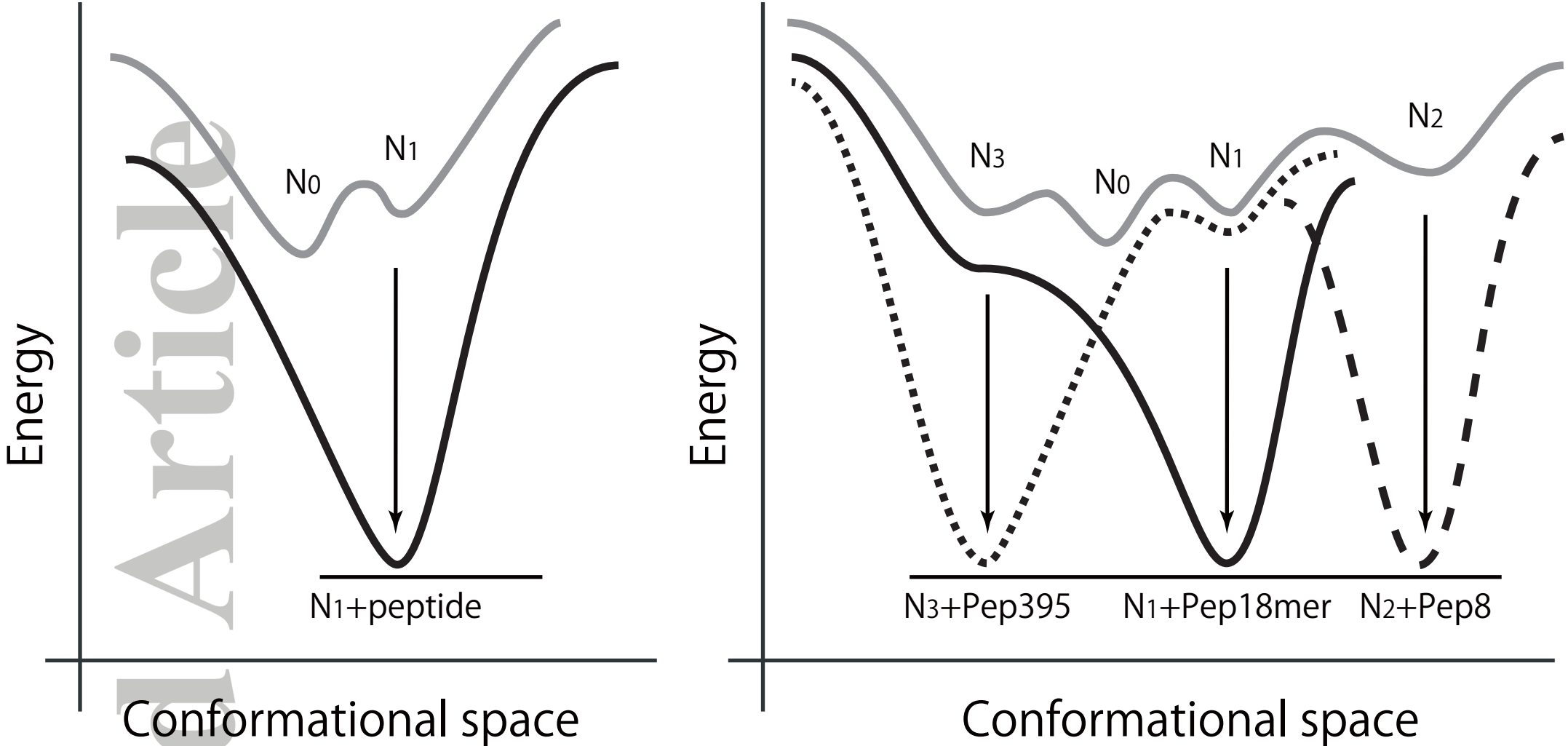




Mahmud et al., Figure 2



Mahmud et al., Figure 3



Mahmud et al., Figure 4