Title: Supercomplex formed by the cross-binding of multiple antibody-single repeat chain complex gives signal amplification of antibody based assay

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Research Article

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Abstract

It is a challenging subject of biomedical research to develop more efficient and sensitive assay method. New method for enhancing sensitivity and precision of conventional immunological assays is developed. The antibody binding domain of *Streptococcus* protein G was used to make a chain of repeated antibody binding domain. The repeat chain was mixed with antibody, and multiple number of antibody bound to the repeat chain to form multiple antibody-repeat chain complex. The cross-binding between the complexes formed supercomplex, and the supercomplex amplified signals without specificity loss and background noise increase.

Introduction

There are many immunological assays used in biological research. Western blot and enzyme-linked immunosorbent assay (ELISA) are the typical immunological techniques. These assays are based on specific antigen-antibody binding (*Fig. 1a*). They are dependent on multiple factors such as the number of target antigen, the affinity and specificity of antibody, and the sensitivity of detection method. Improvement in sensitivity of these assays is one of the challenging works of biomedical research. Conventional approaches to enhance the sensitivity of these assays include the enhancement of the efficiency of chemiluminescence substrates or fluorescence chromophore, and the enrichment of the antigen population by immobilized antibody as in sandwich ELISA\(^1\)\(^-\)\(^4\). Most of these approaches have been to enhance the signal strength. However, the increase of signal strength accompanies inevitable increase of background noise, or expensive and sensitive equipment is needed for the detection of signals such as fluorescence. Here, we show a simple way of signal amplification that gives high specificity and high signal to noise ratio and is immediately applicable to conventional immunological assays.

Results

**Repeat Chain of Protein G immunoglobulin Binding Domain.** Domain III of the immunoglobulin binding domains of streptococcal protein G was used to construct tandem repeat (TR) chain. It is known that they bind to Fc and to Fab domain of the antibody. The domain III of the three Ig binding domains from protein G was used to construct up to 10 repeat (TR10) (*Supplementary Fig.1*)\(^5\). As expected from its antibody binding characteristics, we found that TR10 could bind up to three Fab containing molecules. (*Supplementary Fig.2*). In this study, it is expected that increased structural flexibility between the binding domains and increased number of the binding domain in the repeat chain will increase the number of antibodies bound to the repeat chain. As an antibody molecule has flexible bending, free rotating Fab domain, we predicted that TR10 could bind three whole antibody molecules. The whole antibody has two Fab domains and one Fc domain, and it is possible for an antibody to bind two repeat chain simultaneously. This simultaneously binding results in cross-binding between multiple antibody-single repeat chain complex. The cross-binding between the complexes can produce supercomplexes, a large group of cross bound complexes.
**Supercomplex.** The antibody binding domain of protein G is known to bind to Fc and Fab domain of an antibody with different affinity \(^6\). There are 10 antibody binding domains in TR10. It is possible to imagine that the cross-binding between complexes can make super-molecular supercomplex. We hypothesized that the supercomplex may have a snowball like gigantic congregate shape (Fig. 1b). To check our assumption for the supercomplex formation and antibody signal amplification by the TR10, we tested the effect of TR10 on immunological assays. We made the supercomplexes of TR10 and anti-\(\beta\)-actin monoclonal antibody (Pri. Ab). Three different molar ratios of antibody to TR10, 1:1/10, 1:1/5, and 1:1, were used to check the difference of the effect of supercomplex formation depending on the molar ratio. The supercomplex was freshly made simply by mixing TR10 and antibody and incubating at 37°C for an hour, and kept on ice until use.

**Signal Amplification by Supercomplex; western blotting.** Adding TR10 to the western blot assay increased signal more than 15-fold than the conventional way. Clear lysate of A431 human cancer cells and primary and secondary antibody (Santa Cruz, USA) were used. The supercomplex was made freshly each time and at the same concentration of the primary antibody, 1 in 1000 dilution. When we used conventional ECL reagent on nitrocellulose membrane, it was only possible to detect at very high concentration of the lysate. (Data not shown) We tested sensitive Femto luminescence reagent at low lysate concentration (Supplementary Fig.3). It was also tested whether the Supercomplexes give different signal amplification depending on their Ab:TR10 molar ratio. No strong dependency of the signal on molar ratio was observed within the tested.

We used PVDF membrane to compare with nitrocellulose membrane. On nitrocellulose membrane, we could see about 17-fold increase of signal (Fig.2a). With PVDF membrane, we probed first with conventional reagent ECL. We instantly washed the membrane with 1X TBST and the Super signal Femto luminescence reagent (Thermo scientific, USA) was added to get the second western signal. For the third analysis on the same PVDF membrane, the antibodies were stripped out from the membrane and it was reprobed with supercomplex (Ab:TR10=10:1) and ECL reagent (Fig.2b). The supercomplex amplified signal with ECL reagent was comparable to that with Femto reagent. However, huge background noise was observed with femto reagent due to nonspecific adsorption of secondary antibody on PVDF membrane and highly sensitive femto reagent. Supercomplex can increase the sensitivity of the conventional reagent on PVDF about 15-fold and it is similar to the increase observed on NC membrane. With the conventional ECL reagent, we could see high performance of PVDF membrane compared to NC membrane with Femto reagent. In the densitometry analysis, the correlation between the signal amplification and molar ratio was not clearly observed because the band intensity on the film got saturated. Because of the saturation of band intensity on film, there was inherent limit of precise measurement of signal in the densitometry analysis (Supplementary Fig.4).

**ELISA; Quantitative analysis of signal amplification of the Supercomplex.** The effect of supercomplex was tested on ELISA. We set up an indirect ELISA that detects \(\beta\)-actin coated on the 96-well plate. The spectroscopic measurement gave very accurate quantitative result (Fig.3a). With 1:60 dilution of primary
antibody, the supercomplex was prepared at primary antibody to TR10 molar ratio of 1:1/10, 1:1/5 and 1:1, and gave 5.3, 13.3, and 21.9 fold increase over the conventional method.

The binding of the secondary antibody is through primary antibody, and the intensity of detected signal is dependent on how many primary antibodies are bound. At the very high dilution (1:3000) of primary antibody, the increase of secondary antibody did not give differences (Supplementary Fig.5). With the dilution of primary antibody, the signal amplification decreased regardless of the molar ratio (Fig.3b). It seems that the amplification effect by supercomplex disappeared when the amount of primary antibody is far less than that of antigen (Fig.3b, 1:3840 dilution). The increase of A450 was higher at high primary antibody concentration at all molar ratio. The formation of supercomplex is dependent on the primary antibody concentration and the molar ratio of antibody and TR10. At the 1:30 dilution of primary antibody, the supercomplex formed at 1:1/10 molar ratio showed 10.2-fold increase, but the supercomplex at 1:1/5 molar ratio gave too large signal over the limit of ELISA reader (Supplementary Fig.6). With 1:120 dilution of primary antibody, the antigen was serially diluted to see the signals at low antigen concentration (Fig.3c). The addition of TR10 gave higher signal level at all tested antigen concentration than without TR10, and amplified signals depending on the antigen concentration showed large differences whereas the conventional method gave marginal differences. The signal increases of 1:1 molar ratio was highest at all antigen concentration and it gave the highest increase at high antigen concentration (Fig.3d). The average of the signal increase at molar ratio of 1:1/10, 1:1/5, and 1:1 are 3.983, 6.950, and 14.48 fold, respectively. We think that they are dependent on the number of primary antibody on the surface of supercomplex that is accessible to the secondary antibody. The formation of supercomplex is assumed to be dependent on the molar ratio, affinity between the antibody and TR10, cross-binding between complexes, steric hindrance and preparation condition. The patent has been applied.7

Discussion

At very high antibody and TR10 concentration, the phase separation of the supercomplex produced precipitation out of the solution. The precipitates could be collected at the bottom of the test tube. The supercomplex formed at low concentration did not give noticeable precipitate and could be used for the assay. We showed that supercomplex could enhance the sensitivity of conventional western blotting over 17.5 fold. The sensitivity enhancement by supercomplex was without any increase of background noise level, and the enhancement was observed on both of nitrocellulose and PVDF membranes. The supercomplex also significantly amplified the ELISA signal level up to 22.9 fold and gave signals even below the low limit of detection of the conventional ELISA. The supercomplex in this work can be prepared by simply adding the TR10, and it can be widely applicable to biochemical assays based on antigen-antibody binding without the increase of background noise.

Methods
Recombinant tandem repeat proteins of the antibody binding domain of streptococcal protein G (TR10) were prepared according to the method described previously. Primary Antibody and secondary antibody were purchased from Santa cruz biotech, which are mouse anti–beta-actin monoclonal antibody (sc-47778) and horse radish peroxidase conjugated goat anti-mouse antibody. A431 or AGS human gastric cancer cell line was used for preparing cell lysate. Cultured cells were washed with phosphate buffered saline (PBS) and lysed by adding cell lysis buffer (1% IGEPAL CA-630 and 10mM Tris/HCl pH6.8, 150mM NaCl, 1 mM EDTA, 100mM PMSF, and protease inhibitor cocktail from Sigma). Protein concentration was measured by BCA assay reagent (Thermo Scientific). Supercomplex was freshly produced by incubating TR10 and primary antibody for 1 hour at 37°C and kept at 4°C until use.

**Western blotting**

Protein sample for SDS-PAGE was prepared by mixing the cell lysate with SDS sample buffer. SDS-PAGE was done with Bio-Rad mini gel and proteins were transferred to nitrocellulose or PVDF membrane at 4°C overnight. All the procedure after the membrane transfer was done at room temperature. Membrane was washed briefly with appropriate volume of tris-buffered saline/ 0.1% (v/v) Tween-20 (TBS/T). Membrane blocking was done with 2% (w/v) chicken serum albumin in TBS/T for 1 hr. Incubation for primary and secondary antibody was done in blocking buffer with gentle agitation for 1 h. The dilution factor of the HRP-conjugated secondary antibody was 1:2000 unless otherwise indicated. The membrane was washed three times for 5 minutes each with 15 ml of TBS/T after primary and secondary antibody incubation. Membrane was incubated with luminol substrate solution prepared (100 mM Tris/HCl pH 8.8, 1.25 mM luminol, 2 mM 4IPBA, 5.3 mM hydrogen peroxide) or Super signal femto maximum sensitivity reagent (Thermo scientific) and immediately exposed to x-ray film.

**Direct ELISA**

Clear lysate of AGS human gastric cancer cells was diluted to a final concentration of 20μg/ml total protein in 100mM carbonate coating buffer pH9.6. Each well of a microplate was coated with 1μg lysate and incubated overnight at 4°C. The rest of the procedures were carried out at room temperature. The remaining protein-binding sites in the coated wells were blocked by adding 200μl blocking buffer, 1% BSA in PBS with 0.05% (v/v) Tween-20 (PBST), per well and incubated for 2 hr. Antibody incubation for both of primary and secondary antibody was done by adding 100μl of antibody solution diluted at the indicated concentration in blocking buffer for 1 hr. Between every step, the plate was washed trice with 200μl PBST. The solutions or washes are removed by flicking the plate over a sink. The remaining drops are removed by patting the plate on a paper towel. For detection, each well was added with 100 μl of the TMB substrate solution. After sufficient color development (30 minutes), stop solution (2M H₂SO₄) was added to the well 100μl each. The optical density of each well was read by a plate reader (Bio-Rad).

**Declarations**

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Author contributions

These authors contributed equally: Yongchan Lee, SunMin Kim

Y.L. and M.C. conceived the described method.

Y.L., S.K. designed and performed laboratory work and interpreted the data.

J.P. contributed with the construction and purification of TR10 Protein.

Y.L., S.K. and M.C. wrote the manuscript and S.K. finalized the draft.

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Figures
Figure 1

Schematic description of antigen detection in immunological assay and the formation of supercomplex. 

a, Conventional in vitro antigen detection depicted in this figure is a basic way of biochemical analysis such as western blotting and ELISA. b, Proposed description of supercomplex.
Figure 2

Supercomplex enhanced the chemiluminescence signal of western blot. Supercomplex was prepared with the molar ratio, that is indicated in parenthesis. The primary antibody was mouse monoclonal anti-β-actin antibody (Santa Cruz Biotech). The secondary antibody, goat anti-mouse-HRP conjugate was used for both experiments. Primary and secondary antibody were incubated for an hour at RT. a, The A431 clear lysate samples were applied on the same 10 % denaturing SDS-PAGE and transferred to nitrocellulose membrane, which was cut into three pieces and probed with or without TR10. Supersignal Femto substrate (Thermo) was used. b, The samples were separated on 10 % denaturing SDS-PAGE and transferred to PVDF membrane. The membrane was probed with primary antibody alone and conventional ECL and instantly washed with TBST and reprobed with Supersignal Femto substrate. Subsequently, the primary antibodies on membrane were stripped by SDS and 2-mercaptoethanol treatment. The stripped membrane was reprobed with the supercomplex and ECL. ECL was prepared by the method of Haan and Behrmann (2007).

| Lysate ratio: | 512  | 51.2 | 25.6 | 12.8 |
|--------------|------|------|------|------|
| A431 Lysate (µg): | 20   | 2    | 1    | 0.5  |
| Pri. Ab alone | RI: 33.4  | 6.1 | 1 | - |
| Pri. Ab:TR10 (1:1/5) | RI: 27.2 | 17.5 | 7.0 |
| Pri. Ab:TR10 (1:1/10) | RI: 29.0 | 17.3 | 9.0 |
| b. PVDF with ECL or Femto reagent |
| 128  | 64  | 8   | 1 |
| 5  | 2.5 | 0.315 | 0.039 |
| Pri. Ab alone / ECL |
| RI: 1 |
| Pri. Ab alone / Supersignal Femto |
| Ab stripping and reprobing |
| Pri. Ab:TR10 (1:1/10) / ECL |
| RI: 1.8 |

*RI: Relative Intensity of band.
The weakest band was taken as the reference point of each panel.
Figure 3

Supercomplex amplified the signal of ELISA. a, 1g of AGS cell lysate was coated in each well. The primary antibody was serially diluted with or without TR10. means ± s.d. N = 3 b, A450 value of each primary Ab dilution with TR10 was divided by that of primary Ab alone value. The fold increase of absorbance is plotted with the molar ratio of antibody to TR10. c, The AGS cell lysate was serially diluted and coated. The primary antibody was used at xed 1:120 dilution. means ± s.d. N = 3 d, The statistical analysis for fold increase of A450 was done by student t test. ns: p > 0.05, **: p ≤ 0.01, ***: p ≤ 0.001. Each number is the average signal increase of all the antigen lysate concentration at each molar ratio.
Supplementary Files

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- 4.SupplementaryMaterial.pdf