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Research paper

Evaluation of semi-homogeneous assay formats for dual-specificity antibodies

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ABSTRACT

Dual specificity antibodies such as bispecific and Dual Action Fab (DAF) antibodies are emerging therapeutic products with powerful therapeutic potential. New bioassay formats are needed in order to monitor their dual biological activities. Here we describe the optimization and development of a "bridging" binding method in semi-homogenous (SH) assay format for a bi-specific antibody. In the SH assay format, the antigen and secondary antibodies are mixed directly with the sample solution. The bound complex is then captured onto a microtiter plate coated with the other antigen and subsequently detected by colorimetric methods. We demonstrated that an SH assay achieved comparable dynamic range, quantitation, and specificity to the conventional assay format where each reagent is added sequentially followed by separate washing and incubation steps. The SH assay requires fewer wash steps and the overall assay time is shortened by half, which translates to improved efficiency without any requirement for new equipment and reagents. Using the SH assay format, we also demonstrated that a DAF antibody which can bind to two different antigens with either arm could bind both antigens simultaneously *in vitro*. The SH assay format has broad application as an assay platform for assessing antigen binding properties efficiently.

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1. Introduction

Cancer and certain infectious diseases are usually characterized by genetic heterogeneity and are often difficult to treat with a single therapeutic agent. Therefore, combination therapies are widely deployed based on the prevailing "one antibody-one antigen" dogma. In order to improve the therapeutic utility of antibodies, different types of mAbs have been created in recent years with more powerful therapeutic potential (Herold, 2010; Hollander, 2009; Hudson and Souriau, 2003). One of these is the generation of dual specificity antibodies such as bispecific and dual action Fab antibody which can, by virtue of combining two binding specificities, improve the efficacy of antibody-based treatment of human disease (Deyev and Lebedenko, 2008; Baeuerle and Reinhardt, 2009; Nelson et al., 2010).

Bispecific antibodies (bsAbs) capable of simultaneously binding to two different targets shown in Fig. 1A are composed of two different heavy/light chains. Each arm of the bispecific antibody only binds to one specific antigen. Efficient heterodimerization of the two specific chains of bsAbs can be achieved through the "knob-in-hole" engineering of CH3 domains (Xie et al., 2005) (Fig. 1A). The Dual Action Fab (DAF) antibody, on the other hand, is a "two-in-one" designer antibody, where the same binding site on a regular antibody is engineered to recognize two different antigens, both with high affinity (Bostrom et al., 2009). The DAF antibody is composed of two identical heavy chains and light chains. Unlike in a bispecific antibody where each arm binds to a different antigen, both arms of a DAF antibody can bind to either antigen (Fig. 1B).

In this paper, we will describe the development and optimization of a "bridging" binding method and implementation of a semi-homogeneous (SH) assay format for a recombinant human bispecific antibody. We also applied this SH assay format to a DAF antibody and demonstrated that a DAF antibody could bind two different antigens simultaneously *in vitro*.

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2. Materials and methods

2.1. Materials

Recombinant humanized IgG1 bispecific MAb1 and Dual Action Fab (DAF) MAb2 were generated at Genentech (a member of the Roche group, South San Francisco, CA).

2.2. Biotinylation of antigen C

Antigen C was biobinylated through amine coupling using No-Weigh Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL) according to the manufacture's protocol at a nominal 5:1 molar ratio of biotin to antigen C.

2.3. Assay procedures

2.3.1. Conventional assay format (serial addition assay format)

Briefly, 96 well Maxisorp immunoplates were coated with antigen B overnight at 4 °C. The following day, the plates were blocked with 200 μl/well blocking buffer (0.5% bovine serum albumin [BSA] in PBS) followed by addition of 100 μl of MAb1 serially diluted at 1:2 in assay diluent (PBS, 0.5% BSA and 0.05% P20) to the plate in duplicate wells. The plate was incubated at room temperature with agitation for 1–2 hours, washed with wash buffer (PBS, 0.05% P20) three times, followed by the addition of 100 μl of 8 μg/ml antigen A and then incubated for 1–2 hours. The plate was then washed three times followed by the addition of 100 μl horseradish peroxidase (HRP) conjugated anti-His and incubated for 1–2 hours. Finally, the plates were washed and the HRP substrate 3,3′,5,5′-tetramethylbenzidine (KPL, Inc; Gaithersburg, MD) was added to the wells. Color development was stopped by the addition of 0.6 N sulfuric acid and absorbance was measured at 450 nm, with reference at 650 nm using a SpectraMax plate reader (Molecular Devices; Sunnyvale, CA). Results, expressed in optical density (OD), were plotted against the antibody concentrations using a 4-parameter curve fitting program.

2.3.2. Semi-homogeneous assay format

Briefly, 96 well Maxisorp immunoplates were coated with antigen B overnight at 4 °C. The following day, the plate was blocked with 200 μl/well blocking buffer before a mixture of MAb1, antigen A and anti-His HRP was added to the plate. The plate was incubated at room temperature with agitation for 1–2 hours, the plate was then washed and the HRP substrate 3,3′,5,5′-tetramethylbenzidine (KPL, Inc; Gaithersburg, MD) was added to the plates. Color development was stopped by the addition of 0.6 N sulfuric acid and absorbance was measured at 450 nm, with reference at 650 nm using a SpectraMax plate reader (Molecular Devices; Sunnyvale, CA). Results, expressed in optical density (OD), were plotted against the antibody concentrations using a 4-parameter curve fitting program.

3. Results and discussion

3.1. Evaluation of SH assay formats for a bispecific antibody

Each Fab arm of the bispecific MAb1 binds to a different antigen A or B (Fig. 1A). Therefore, we developed a "bridging" binding assay in which binding to both antigen A and B can be measured (Fig. 2, panel A). Conventionally, when running the assay, reagents are added to the plate sequentially, with each addition separated by a wash step. Typically an assay would require approximately 4 hours from blocking to reading the plate. In order to shorten the assay time, we evaluated the SH assay format (Fig. 2, panel B), in which most of the required reagents (e.g., antigen A, detection antibodies) are mixed directly with the sample solution, allowing components to be efficiently bound together in solution. The bound complex is then specifically captured onto the plate surface and subsequently detected by colorimetric methods. The SH assay format shortens the overall assay time by half.

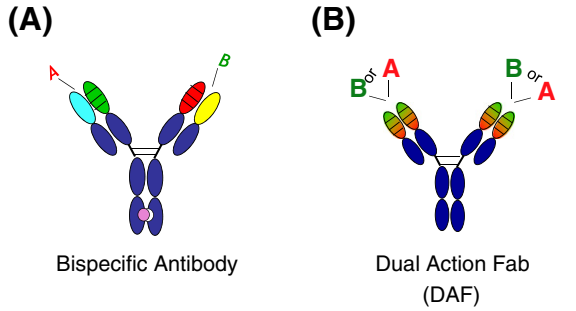

Fig. 1. Engineered formats of "knob-in-hole" bispecific antibody (A) and dual action Fab antibody (B). Both types of antibody can bind two different antigens A and B.

3.2. Optimization of the SH assay format

During the initial SH assay evaluation, a decrease in signal was observed at high concentration of MAb1 (Fig. 3A). This "hook effect" was thought to be due to inadequate amount of antigen A, i.e. at high concentration of MAb1, the antibody may start to have only one arm binding to either antigen A or B, instead of both arms being occupied by both antigen A and B. Thus, it is crucial to determine the saturation concentration of antigen A for the maximum amount of antibody used in the assay. To assess that, various concentrations of antigen A were evaluated while keeping the concentration of MAb1 and anti-HIS-HRP fixed at 4ug/ml and 0.15U/ml separately. As shown in Fig. 3B, about 8 μg/ml antigen A was needed to saturate the binding to 4ug/ml MAb1 in the homogeneous mixture. At this concentration, all antibodies in the homogeneous solution would be bound with antigen A regardless whether it is bound to the plate or not.

By optimizing antigen A concentration in the homogeneous mixture, the "hook effect" was decreased (data not shown); however, a small "hook effect" was still present. This dip in signal was speculated to be the result of sub-optimal anti-HIS-HRP concentration. Just as for antigen A binding to the antibody, the levels of anti-HIS-HRP bound to antigen A should also be saturating. Therefore, various concentrations of anti-His-HRP were evaluated while keeping the concentration of MAb1 and antigen A fixed. The saturating anti-His-HRP concentration was determined to be 0.3 U/ml and no "hook effect" was observed (Fig. 4A). The binding curve was comparable with assays run with the conventional method (sequential addition of reagents) (Fig. 4B). With the SH assay