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An efficient process of generating bispecific antibodies via controlled Fab-arm exchange using culture supernatants

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ABSTRACT

Bispecific antibody generation is actively pursued for therapeutic and research antibody development. Although there are multiple strategies for generating bispecific antibodies (bsAbs); the common challenge is to develop a scalable method to prepare bsAbs with high purity and yield. The controlled Fab-arm exchange (cFAE) method combines two parental monoclonal antibodies (mAbs), each with a matched point mutation, F405L and K409R in the respective CH3 domains. The conventional process employs two steps: the purification of two parental mAbs from culture supernatants followed by cFAE. Following a reduction/oxidation reaction, the bispecific mAb is formed with greater than 95% heterodimerization efficiency. In this study, cFAE was initiated in culture supernatants expressing the two parental mAbs, thereby eliminating the need to first purify the parental mAbs. The bsAbs formed in culture supernatant was then purified using a Protein A affinity chromatography. The BsAbs generated in this manner had efficiency comparable to the conventional method using purified parental mAbs. BsAbs prepared by two different routes showed indistinguishable characteristics by SDS capillary electrophoresis, analytical size exclusion, and cation exchange chromatography. This alternative method significantly shortened timelines and reduced resources required for bsAb generation, providing an improved process with potential benefits in large-scale bsAb preparation, as well as for HTP small-scale bsAb matrix selection.

1. Introduction

While antibodies have been used extensively as diagnostic and therapeutic agents, there is an increased demand to generate bispecific antibodies (bsAbs) that can bind to at least two antigens. Such bispecific antibodies introduce applications which can include activity on multiple pathways, simultaneous target engagement to increase specificity and selectivity, tissue targeting, and directed cellular toxicity [1]. There are extensive efforts to generate bsAbs, which include having an antibody with dual specificity in a single Fab-arm [2–4], quadroma expression in hybridomas [5–7]; co expression of relevant heavy and light chains [8–21]; chemical crosslinking [22,23]; two in one antibodies [24], dual variable domain antibodies [25]. A common drawback of these methods is the difficulty in producing human bsAbs with high yield and high efficiency without the development of customized methods for preparation.

Controlled Fab-arm exchange (cFAE) is a robust method to prepare bsAbs that easily integrates platform methods developed for regular mAbs [26,27]. The process involves combining two purified parental mAbs each with matched point mutations, F405L or K409R in the respective CH_3 domains. These mutations allow the homodimeric, monospecific parental mAbs against different targets to recombine to form a single heterodimeric, bispecific antibody following a reduction/oxidation reaction in the presence of a reducing agent such as 2-MEA. This process results in generating bsAbs with a greater than 90% heterodimerization efficiency and

Abbreviations: Ab, Antibody; mAb, Monoclonal antibody; bsAb, Bispecific antibody; bsAb Pur, Bispecific antibody generated from purified parental mAbs; bsAb Sup, Bispecific antibody generated in protein culture supernatants; cFAE, Controlled Fab-arm exchange; CIEX, Cation exchange chromatography; HIC, Hydrophobic interaction chromatography; SE-HPLC, Size exclusion high pressure liquid chromatography; cSDS, capillary SDS electrophoresis; HTP, High-throughput; 2-MEA, 2-Mercaptoethylamine-HCl.

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greater than 90% yield [27].

The established cFAE method involves Protein A affinity purification of each parental mAb followed by buffer exchange into phosphate buffer at physiological pH conditions. The parental mAbs are then mixed and incubated with a reducing agent to initiate the Fab-arm exchange process. Following the reduction process, the reducing agent is removed by dialysis allowing re-oxidation to form bsAbs.

In this study, the process could be accelerated by conducting the cFAE prior to purification of the parental human IgG1/Kappa mAbs with the respective F405L and K409R mutations. Thus, cFAE was implemented using culture supernatants from separate cultures that were producing the respective parental mAbs. The bsAbs formed in the combined culture supernatants were then purified using a single Protein A affinity purification step. We demonstrate how such bsAbs were identical in biophysical properties and heterodimerization efficiency to bsAbs generated by the conventional cFAE method using purified parental mAbs.

2. Materials and methods

2.1. Materials

The MabSelect SuRe Protein A resin and HiTrap MabSelect Sure Protein columns were purchased from GE Healthcare (Uppsala, Sweden). The TSKgel BioAssist G3SWxl column was purchased from TOSOH (Part#20026, King of Prussia, PA) and ProPac WCX-10 analytical column from Dionex (Thermo scientific, Sunnyvale, CA). The Butyl-NPR column was purchased from TOSOH (Part# 42168, King of Prussia, PA). 1X DULBECCO'S phosphate buffer saline (D-PBS, pH 7.2) was purchased from Gibco (Grand Island, NY). Sodium Acetate was purchased from Mallinckrodt (Paris, KY), Glacial Acetic Acid from Aqua Solutions, Tris from MP Biomedicals (Illkirch, France), Sodium Phosphate Monohydrate from JT Baker (Phillipsburg, NJ), Sodium Chloride and 2-Mercaptoethyamine-HCl (2-MEA) were procured from Sigma (St. Louis, MO).

2.2. Expression of antibodies

CHO K1SV, a Chinese hamster ovary (CHO) cell line, was grown in chemically-defined media and was transfected with an expression vector containing the genes encoding the light chain (LC) and heavy chain (HC) of the human IgG1/Kappa antibody molecules with the respective F405L and K409R mutations. The stable cell lines were generated, expanded, and maintained in a humidified incubator at 37 °C and 5% CO_2 using tissue culture plates and shake flasks. Cells were grown for immunoglobulin production in bolus fed-batch shake flasks. Culture supernatants were harvested by centrifugation and further clarified by filtration.

2.3. Antibody expression level determination

To determine the antibody expression level in the culture supernatant, two methods were used: a batch purification method using loose MabSelect SuRe Protein A resin; and a method using 1 mL HiTrap MabSelect SuRe column. In the first method, MabSelect SuRe Protein A resin was washed in water and resuspended in D-PBS pH 7.2. 10 mL of each culture supernatant was added to 2 mL of resin. The mixture was rotated at room temperature for 30 min. Unbound protein was removed by centrifugation at 1000 rpm for 10 min. Resin bound protein was suspended in 10 mL D-PBS and centrifuged at 1000 rpm for 10 min. The supernatant was carefully removed to minimize resin bed disturbance. This step was repeated three times. To elute the bound protein, resin was then suspended in 5 mL 0.1 M Sodium Acetate pH 3.5 and centrifuged at 1000 rpm for 10 min. The supernatant was gently drawn using a syringe, 0.22 μM filtered, neutralized with 1 mL 2.5 M Tris pH 7.2.

In the second method, columns were equilibrated with 5 column volumes (CV) of D-PBS, pH 7.2 prior to loading 10 mL of each culture supernatants. Unbound proteins were removed by washing with 10 CV D-PBS, pH 7.2. Bound protein was eluted with 5 CV 0.1 M Na-acetate, pH 3.5 at 1 mL/min. Peak fractions were neutralized with 10% 2.5 M Tris pH 7.2 and pooled.

The protein concentration for each elution pool was determined by measuring absorbance at OD280 nm on a NanoDrop1000 spectrophotometer and calculated using the 280 nm absorbance extinction coefficient based on the amino acid sequence.

2.4. Controlled Fab-arm exchange in culture supernatant

Culture supernatants expressing recombinant mAb A or mAb C with F405L and recombinant mAb B or mAb D with K409R were mixed at a 1:1 molar ratio based on the protein concentration in culture supernatant determined as described above. 2-MEA dissolved in D-PBS was added to a final concentration of 75 mM. The mixture was rotated for 5 min at room temperature and then transferred to a 31 °C incubator where exchange was allowed to continue for 5 h without agitation.

2.5. Purification of bsAbs and parental antibodies

The Fab-arm exchanged culture supernatant mixture, and individual culture supernatants expressing mAb A, mAb B, mAb C and mAb D parental Abs were purified using pre-packed 1 m or 5 mL HiTrap MabSelect SuRe Protein A columns. Columns were equilibrated with 5 column volumes (CV) of D-PBS, pH 7.2 prior to loading the culture supernatants. Unbound proteins were removed by washing with 10 CV D-PBS, pH 7.2. Bound protein was eluted with 5 CV 0.1 M Na-acetate, pH 3.5 and neutralized with 2.5 M Tris pH 7.2. The neutralized protein solution was dialyzed into D-PBS, pH 7.2.

2.6. Fab-arm exchange using purified parental mAbs

The two purified parental mAbs were mixed in a 1:1 molar ratio in D-PBS. 2-MEA dissolved in D-PBS was added to a final concentration of 75 mM. The mixture was rotated for 5 min at room temperature before being transferred to a 31 °C incubator where the exchange was allowed to continue for 5 h without agitation. The 2-MEA was removed by dialysis, using a SLIDE-A-LYZER to a targeted final concentration of below 50 μM.

2.7. Analytical characterization of purified bsAb and parental mAbs

The protein concentration for each purified mAb was determined by measuring the absorbance at 280 nm on a NanoDrop1000 spectrophotometer and calculated using the extinction coefficient based on the amino acid sequence.

SE HPLC of the purified antibodies was performed by running samples on a TOSOH TSKgel BioAssist G3SWxl column, in 1X D-PBS, pH 7.2 at 1 mL/min on a Waters Alliance HPLC for 20 min. 20 μg of each protein was injected on to the column. The column effluent was monitored by absorbance at 280 nm. The output was integrated using the Millenium/Empower software.

Parental and bsAbs were analyzed under reducing and non-reducing alkylating conditions on the Caliper LabChip GXII analytical separation instrument. To prepare the reduced samples, five microliters of each protein at a concentration of 1–2 mg/mL in D-PBS was added to a well in a 96-well plate (Hard-shell PCR Plate, Bio-Rad). Thirty five microliters of reducing sample buffer [10X