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Research paper

A novel efficient bispecific antibody format, combining a conventional antigen-binding fragment with a single domain antibody, avoids potential heavy-light chain mis-pairing

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ABSTRACT

Due to the technical innovations in generating bispecific antibodies (BsAbs) in recent years, BsAbs have become important reagents for diagnostic and therapeutic applications. However, the difficulty of producing a heterodimer consisting of two different arms with high yield and purity constituted a major limitation for their application in academic and clinical settings. Here, we describe a novel Fc-containing BsAb format (Fab × sdAb-Fc) composed of a conventional antigen-binding fragment (Fab), and a single domain antibody (sdAb), which avoids heavy-light chain mis-pairing during antibody assembly. In this study, the Fab x sdAb-Fc BsAbs were efficiently produced by three widely used heavy-heavy chain heterodimerization methods: Knobs-into-holes (KIH), Charge-pairs (CP) and controlled Fab-arm exchange (cFAE), respectively. The novel Fab x sdAb-Fc format provided a rapid and efficient strategy to generate BsAb with high purity and a unique possibility to further purify desired BsAbs from undesired antibodies based on molecular weight (MW). Compared to conventional BsAb formats, the advantages of Fab x sdAb-Fc format may thus provide a straightforward opportunity to apply bispecific antibody principles to research and development of novel targets and pathways in diseases such as cancer and autoimmunity.

1. Introduction

Bispecific antibodies (BsAbs) have been considered promising cancer therapeutics for a long period of time. The first artificial antibody-based molecule with the ability to bind to two different antigens at the same time was described by Nisonoff's team in 1960s, which marked the launch of a long BsAb generation campaign (Nisonoff et al., 1960). Compared to monospecific monoclonal antibodies, the potential advantages of BsAbs are undisputed. When applied to cancer therapy, BsAbs have shown potential to redirect specific immune cells to tumour cells to enhance tumour killing (Staerz et al., 1985). Furthermore, given that cancer is a complex, multifactorial and heterogenic disease involving many disease-driving proteins and cross-talking pathways, BsAbs can be used to target two antigens that each are not necessarily tumour-specific but targeting the combination improves selectivity of tumour targeting over normal tissues (Mazor et al., 2015a) (Mazor et al., 2017). Moreover, dual targeting could be useful to modulate two separate functional pathways in the tumour, or to avoid resistance to the treatment (Lopez-Albaitero et al., 2017) (Moores et al., 2016).Nevertheless, BsAbs have not yet stimulated broad interest of pharmaceutical companies until recent years due to challenges in BsAb manufacturing (Garber, 2014). By the end of 2017, compared to the number of 57 clinically approved mAbs, there were only two BsAbs on the market (Grilo and Mantalaris, 2019). The major challenge in the development of BsAbs is the difficulty in producing a pure BsAb without the presence of contaminating antibody by-products such as non-functional or

Abbreviations: ADCC, Antibody-dependent cell-mediated cytotoxicity; ADCP, Antibody-dependent cellular phagocytosis; BsAb, Bispecific antibody; CDC, Complement-dependent cytotoxicity; CE-SDS, Capillary electrophoresis sodium dodecyl sulfate; cFAE, controlled Fab-arm exchange; cIEF, Capillary isoelectric focusing; CIEX, Cation exchange chromatography; CP, Charge-pairs; DB, Duobody; Fab, Antigen binding fragment; Fc, Fragment crystallizable; HcAb, heavy chain only antibody; HIC, hydrophobic interaction chromatography; HP-SEC, High performance size-exclusion chromatography; IgG, Immunoglobulin G; KIH, Knobs-into-holes; LC-MS, Liquid chromatography–mass spectrometry; mAbs, monoclonal antibodies; MW, molecular weight; pI, isoelectric point; sdAb, Single domain antibody.

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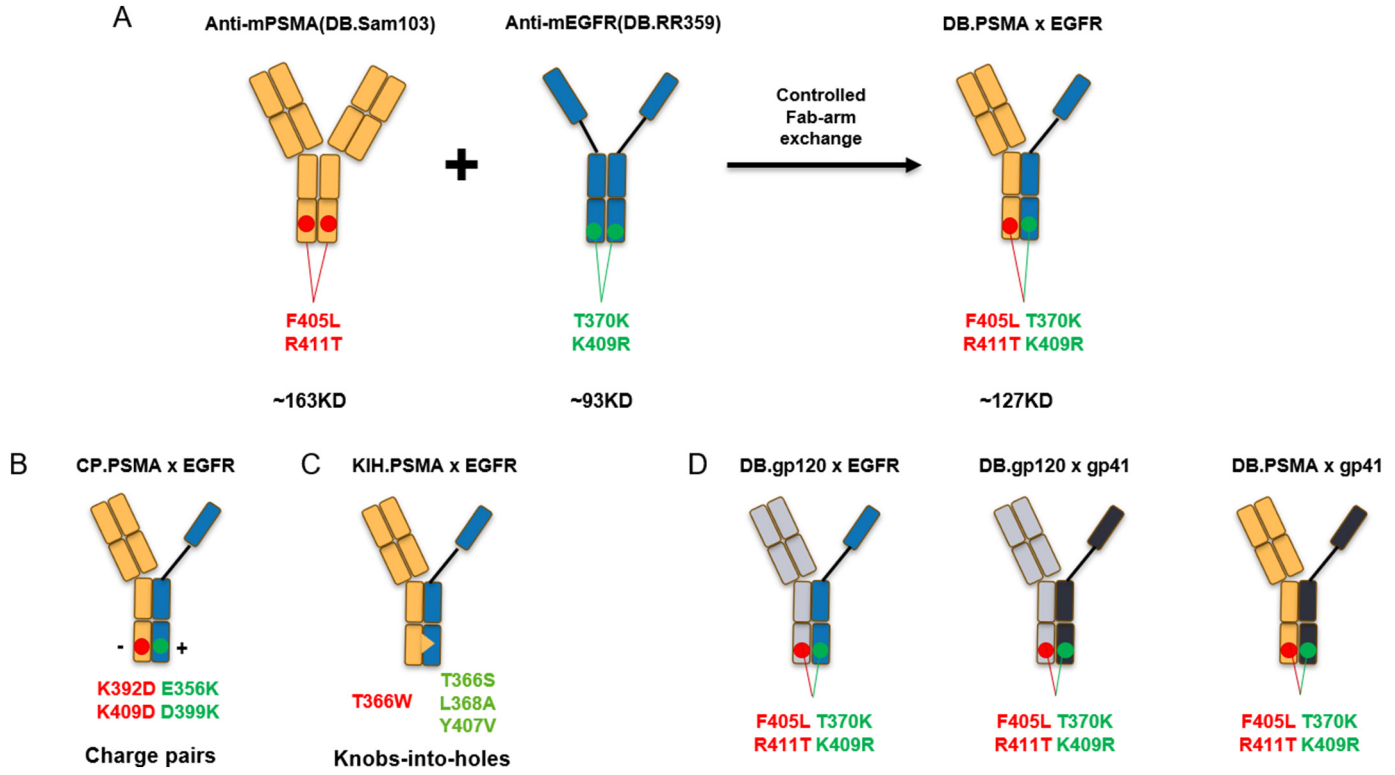


Fig. 1. Schematic diagram of mouse IgG2a-based Fab x sdAb-Fc BsAbs generated by using (A) controlled Fab-arm exchange (cFAE), (B) charge-pairs (CP) and (C) knobs-into-holes (KIH); (D) Design of bispecific control antibodies using the cFAE method.

monospecific molecules formed during assembly.

Over the past two decades, with the development of protein and gene engineering, over 100 different formats of BsAbs have become available which fall into four classes: Fc containing asymmetric architecture, Fc-less asymmetric architecture, Fc containing symmetric architecture and Fc-less symmetric architecture (Brinkmann and Kontermann, 2017) (Ha et al., 2016). The diversification of BsAb formats allows researchers to modify the size, half-life, valency, flexibility, and biodistribution of BsAb for applications of different purposes. In many cases, the Fc region is needed to ensure a relatively long pharmacokinetic in vivo half-life and the ability to induce secondary immune functions of a given BsAb, such as Antibody-dependent cell-mediated cytotoxicity (ADCC), Antibody-dependent cellular phagocytosis (ADCP) and Complement-dependent cytotoxicity (CDC). To form a Fc containing asymmetric BsAb, several methodologies have been developed to enforce the correct association of the two different heavy chains of a BsAb during cellular expression. Well-known examples are knobs-into-holes (KIH) (Merchant et al., 1998) (Atwell et al., 1997), charge-pairs (CP) (Gunasekaran et al., 2010), leucine zipper induced heterodimerization (LUZ−Y) (Wranik et al., 2012), strand-exchange engineered domain CH3 heterodimers (SEEDbody) (Davis et al., 2010), and HA-TF (Moore et al., 2011).

These heterodimerization methods solved the heavy-heavy chain mis-pairing problem to a great extent, but inadvertent mis-pairing of heavy-light chain still remained a major limitation. One straightforward method to overcome heavy-light chain mis-pairing is to share common light chain by two different heavy chains (Merchant et al., 1998) (Jackman et al., 2010) (Krah et al., 2017). The latter approach provides a challenge when using pre-existing and validated ('benchmark') parental monospecific antibodies, and parental candidates must be (re-) generated to fit the common light chain approach. Alternatively, to enforce the correct heavy-light chain pairing, some approaches introduced amino acid mutations at the contact points of VH/VL, CH1/CL or both (Igawa et al., 2010) (Lewis et al., 2014) (Bönisch et al., 2017); some approaches exchanged the VH-VL or the CH1-CL domains by domain crossover between the heavy and light chain Fab domains such as CrossMab (Schaefer et al., 2011), DuetMab (Mazor et al., 2015b) and orthogonal Fab (Lewis et al., 2014). Alternatively, the heavy-light chain mis-pairing can be circumvented by producing the BsAb out of two parental mAbs as demonstrated for the Duobody (DB) technology which makes use of controlled Fab-arm exchange (cFAE) methodology to achieve correct heavy-heavy chain heterodimerization (Labrijn et al., 2013).

Importantly, in addition to introducing enhanced immunogenicity risk the extensive protein engineering required for most of these approaches to improve physicochemical, biological characteristics and even affinity lead to the requirement for additional analytical and quality testing (Atwell et al., 1997) (Masuda et al., 2006) (Chailyan et al., 2011) (Herold et al., 2017).

In this study, we describe a novel format of BsAb, the Fab x sdAb-Fc, which combines a conventional antigen-binding fragment (Fab) with a single domain antibody (sdAb), both linked to Fc domains optimized for heavy-heavy chain heterodimerization. This novel format avoids the issue of heavy-light chain mis-pairing and can be used in combination with common heavy-heavy chain heterodimerization strategies. As a proof of concept, we generated BsAbs, in a mouse IgG2a format, specific for two tumour antigens, mEGFR and mPSMA, using well established cFAE, CP and KIH heavy-heavy chain heterodimerization methods. Since the sdAb domain does not bind light chains, the expressed light chain region of the BsAb can only associate with its corresponding heavy chain. Our results show that the Fab x sdAb-Fc BsAb can be generated with high purity, and the products are stable by all quality testing applied suggesting that this novel BsAb format constitutes a convenient and promising technology for exploration of bispecific concepts in the potential treatment of cancer, autoimmune, inflammatory and other diseases.

2. Materials and methods

2.1. Design of vectors

The anti-mEGFR sdAb (RR359) has been described before (Zaiss et al., 2013) and the amino acid sequence of anti-mPSMA antibody (Sam103) was obtained from patent US20170342169A1. The sequence of isotype control antibodies was obtained from the protein data bank, which PDB IDs were 2NY7 (B12, anti-gp120) and 4B50 (2H10, anti-gp41), respectively. The amino acid mutations introduced to each antibody to allow heavy-heavy chain heterodimerization are depicted in Fig. 1 for the various heterodimerization technologies.

In short, for the Duobody (DB) method using the controlled Fab arm exchange, T370K and K409R point mutations were introduced to the CH3 region of a heavy chain only antibodies, F405L and R411T point mutations were introduced to the CH3 region of the conventional antibodies. Parental mAb expression vectors were constructed by de novo synthesis (GeneArt). For the CP method, E356K and D399K point mutations were introduced to the CH3 region of the heavy chain only antibody, K392D and K409D point mutations were introduced to the CH3 region of the conventional antibody. For the KIH method, T366S, L368A and Y407V point mutations were introduced to the CH3 region of the heavy chain only antibody, T366W point mutation was introduced to the CH3 region of the conventional antibody.

Between the sdAb and the Fc part of the heavy chain only antibody (HcAb), a camelid/mouse chimeric linker was introduced (EPKIPQPQPKPQPQPQPKPQPKPCPPCKCPAPNLLGG). The expression vectors of these antibodies were constructed by de novo synthesis (GeneArt). Amino acid sequences of all antibodies are given in Supplementary Table 1.

2.2. Expression and purification of BsAbs by knobs-into-holes and charge-pairs

The FreeStyle™ 293-F cells (Invitrogen) were grown in FreeStyle 293 Expression medium, (Invitrogen). Each relevant vector (described in Supplementary Table 1) was co-transfected into FreeStyle™ 293-F cells using the 293fectin reagent (Invitrogen) according to the conditions recommended by the manufacturer. 7 days post-transfection, cell suspensions were collected and centrifuged for 15 min at 2500 x*g*. The supernatant was passed through a 0.22 μm filter and stored at 4 °C. The amount of BsAbs in the supernatant was measured by a Cedex bioanalyzer (Roche). The supernatant was then mixed with MabSelect SuRe LX resin (GE Lifesciences) and rotated overnight at 4 °C. After overnight capturing, the BsAbs were purified from the supernatant by affinity chromatography using Pierce™ Centrifuge Columns (ThermoFisher Scientific) and re-buffering to PBS using PD-10 Desalting Columns (GE healthcare) according to the manufacturer's instructions. Antibody concentration was calculated based on Beer-Lambert Law, $A = \varepsilon * b * c$, (A is the A280 absorbance, b is the path length, c is the analyte concentration and ε is the wavelength-dependent molar absorptivity coefficient with units of $M^{-1}\ cm^{-1}$). A280 absorbance of each antibody was measured by spectrophotometry using a Nanodrop ND-1000 system.

2.3. Generation of BsAbs by controlled fab-arm exchange

Parental antibodies were produced under serum-free conditions by co-transfecting relevant heavy and light chain expression vectors in FreeStyle™ 293-F cells, using 293fectin™ according to the manufacturer's instructions. Antibodies were purified by protein A affinity chromatography, dialyzed overnight to PBS, and filter-sterilized over 0.22 μM filters. Antibody concentration was calculated as previously described.

The bispecific antibody was produced by controlled Fab-arm exchange using the two purified bivalent parental antibodies, each with the respective complementary mutations: T370K, K409R or F405L, R411T (specific to mouse IgG2a isotype) (Labrijn et al., 2017). Equimolar amounts of relevant parental antibodies were mixed and incubated with 2-Mercaptoethylamine (2-MEA; Sigma) at a final concentration of 2 mg/mL total antibody in PBS, with the final concentration of 2-MEA being 75 mM. The mixtures were incubated for 5 h at 31 °C. To remove 2-MEA, the mixtures were buffer-exchanged against PBS using Slide-A-Lyzer cassettes (ThermoFisher Scientific). Samples were stored overnight at 4 °C to allow for the re-oxidation of the disulfide bonds. Antibody concentration was calculated as previously described.

Three Fab x sdAb-Fc bispecific control antibodies were designed 1) DB.gp120 x EGFR containing the EGFR monovalent single domain antibody combined with a non-relevant isotype arm (antibody B12, anti-HIV-gp120); 2) DB.PSMA x gp41 containing the PSMA monovalent antibody combined with a non-relevant isotype single domain antibody arm (antibody 2H10, anti-HIV-gp41); 3) Non-functional bispecific DB.gp120 x gp41 containing single-domain antibody arm gp41 combined with arm gp120. The three bispecific control antibodies were produced by cFAE.

2.4. High performance size-exclusion chromatography (HP-SEC)

Aggregation and degradation of BsAbs was quantified by HP-SEC using a YMC-pack Diol-200 column (YMC) with Agilent 1100 series HPLC system. Separation was carried out in 10 mM HEPES, pH 7.4, containing 150 mM NaCl, 3.4 mM EDTA and 0.05% Tween 20.

2.5. SDS-page

Formation of BsAbs was analysed using SDS-PAGE analysis in reducing and non-reducing conditions. Samples were diluted to a final concentration of 0.5 mg/mL with respectively Laemmli Sample Buffer (Bio-Rad) for analysis in non-reducing conditions or Laemmli Sample Buffer containing 200 mM DTT for analysis in reducing conditions. Samples were heated at 99 °C for 5 min. 5 μg of antibody and 10ul Precision Plus Protein All Blue Standards (Bio-Rad) were loaded onto NuPAGE® 4–12% Bis-Tris Gel (Invitrogen). Electrophoresis was run in NuPAGE® MOPS SDS Running Buffer (Invitrogen) at 150 V for 90 min for the non-reduced samples and NuPAGE® MES SDS Running Buffer (Invitrogen) at 150 V for 60 min for the reduced samples. Gels were stained using Coomassie protein assay reagent (Thermo Scientific), destained with distilled water and scanned using ChemiDoc™ Touch Imaging System (Bio-Rad).

2.6. Capillary electrophoresis sodium dodecyl sulfate (CE-SDS)

The purity of all BsAbs were tested by CE-SDS in non-reduced mode. CE-SDS analysis was carried out on a CE system PA800 Plus machine (Beckman Coulter). Samples were diluted to 1 mg/mL with 10 kDa internal standard and 15 mM iodoacetamide in SDS-MW sample buffer and heated to 70 °C for 10 min. 95 μL were transferred into sample vials and loaded into the machine. Separations were performed in a bare-fused silica 50 μm I.D capillary at 22 °C. Effective separation length was 20 cm, run time 30 min and antibody fragments detected at a wavelength of 220 nm. The capillary was flushed with 0.1 M HCl, NaOH, water and running buffer before sample loading at 5 kV for 20 s. Data analysis was carried out with the 32Karat software (version 9.2).

2.7. Capillary isoelectric focusing (cIEF)

cIEF was performed on a Beckman Coulter PA 800 Plus using an amine-coated (eCAP™), 50 μm ID × 30 cm capillary. An ampholyte mixture containing 2.5% (*w/v*) Pharmalyte pH 3–10, 0.2% (w/v) (hydroxypropyl) methyl cellulose, 0.3% (*v*/v) *N,N,N′,N′*-tetramethylethylenediamine, and pI 10 marker was combined with antibody

diluted in water to obtain a final antibody concentration of 0.3 mg/mL. Analysis was performed at 25 kV and 20 °C with a 15 min focusing period under normal polarity during which isoforms migrate to their pI in the pH gradient. This was followed by a 30 min mobilization step using the Bio-Rad chemical mobilizer with the UV detector set at 280 nm.

2.8. Generation of CHO·K1 stable cell line

The CHO·K1 cells (ATCC) were grown in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12), supplemented with 5% New Born Calf Serum (Biowest). 24 μg of pCI-neomEGFR (UniProtKB - Q01279) and pCI-neomPSMA (UniProtKB - O35409) plasmids constructed by de novo synthesis (GeneArt) were transfected into 5×10^6 cells using lipofectamine 2000 reagent (Invitrogen) according to the conditions recommended by the manufacturer. Subsequently, transfected cells were cultured in complete cell culture medium (DMEM/F12 + 5% New Born Calf Serum) with addition of 0.8 mg/ml G418 for 14 days. Selected cells were then stained by rabbit anti-mEGFR-PE (Cell Signalling Technology) or anti-mPSMA antibodies (Sam103), respectively. Positive stained cells were seeded into 96-well plate for clone formation by single cell sorting on a FACSMelody (BD). The clones CHO/mPSMA-HA(A6) and CHO/mEGFR-LA(A1) stably expressed mPSMA or mEGFR, respectively, for more than 2 months and were further used for the present study.

2.9. Cell binding assays

CHO/mEGFR and CHO/mPSMA cells were detached and washed 3 times with FACS buffer (PBS + 1% BSA + 1 mM EDTA). For CHO/mEGFR binding assay, BsAb DB.PSMA x EGFR, BsAb DB.gp120 x gp41 and EGFR HcAb were added in 5-fold serial dilution in FACS buffer, incubated on ice for 45 min. For CHO/mPSMA binding assay, BsAb DB.PSMA x EGFR, BsAb DB.gp120 x gp41 and PSMA mAb were added in 5-fold serial dilution in FACS buffer, incubated on ice for 45 min. Secondary antibody staining was performed using rat-anti-mouse IgG2a antibody conjugated with FITC (Biolegend) in 1:500 dilution in FACS buffer on ice for 45 min. Samples were analysed by FACS Canto™ II (BD) using the software program BD FACSDiva. Ten thousand events were collected.

Flow cytometry was used to determine the simultaneous binding activity of BsAb DB.PSMA x EGFR to mEGFR and mPSMA using stable transfected cell lines (CHO/mPSMA and CHO/mEGFR). In order to prepare single-cell suspension, cells were detached from flasks using cell dissociation buffer enzyme-free, PBS-based (Gibco). Cells were washed cells twice with PBS and CHO/mPSMA and CHO/mEGFR cells were labelled by cell staining dye eFluor 450 or 670 (eBioscience), respectively, according to the conditions recommended by the manufacturer. 5×10^4 cells of cell staining dye eFluor 450 or 670 labelled cell line were mixed in 100 μl FACS buffer (PBS + 1%BSA + 1 mM EDTA) and cells were incubated with bispecific control antibodies (50 μg/ml, equivalent to 390.63 nM) or 5-fold serial dilution of BD.PSMA x EGFR (0.0032 μg/ml to 50 μg/ml, equivalent to 0.005 nM to 390.63 nM) at 4 °C for 45 mins. The stained cells were analysed on a FACS Canto™ II (BD) using the software program BD FACSDiva. Ten thousand events were counted. While the FACS CantoTM II counted every singlet and each cluster as one event, it was unclear from this analysis whether cellular clusters were formed by two cells(doublets), three cells (triplets) or even more, therefore the term “percentage of cellular cluster events” was used to quantify our results.

2.10. Octet

mEGFR-His recombinant protein (R&D systems) was diluted to 50 mM in 10 mM acetate pH 5.0 (ForteBio) and loaded on NHS/EDC activated AR2G biosensors (ForteBio). BsAb DB.PSMA x EGFR and bispecific control antibodies were diluted to 20 μg/mL in 10× kinetic buffer (ForteBio) and associated to mEGFR-His protein. mPSMA-His recombinant protein (Sino Biologicals) was diluted to 50 mM in 10× kinetic buffer and associated to each BsAb. Binding kinetics were measured by Octet system according to the manufacturer's instructions (ForteBio). Data was analysed using Data analysis software HT V10.0 (ForteBio).

3. Results

3.1. Design and expression of BsAbs

To test whether Fab x sdAb-Fc format BsAbs, which combine a conventional antigen-binding fragment (Fab) with a single domain antibody (sdAb) and a mouse IgG2a Fc, can be produced efficiently, we generated Fab x sdAb-Fc BsAbs, using three well-known heavy chain heterodimerization strategies, i.e. cFAE, CP, or KIH, respectively. For the cFAE strategy, parental monospecific bivalent antibodies (T370K and K409R for anti-EGFR antibody RR359; F405L and R411T for anti-PSMA antibody Sam103) were first expressed separately. The BsAb duobody DB.PSMA x EGFR was produced in a second step by cFAE from the purified bivalent parental antibodies (Fig. 1A). In case of the CP and KIH strategies, the CP mutations (K392D and K409D for CH3 domain of anti-PSMA antibody, E356K and D399K for CH3 domain of anti-EGFR antibody) and KIH mutations (T366W for CH3 domain of anti-PSMA antibody, T366S, L368A and Y407V for CH3 domain of anti-EGFR antibody) were introduced into the corresponding expression vectors, respectively. Relevant heavy chain and light chain expression vectors CP mutations or KIH mutations were co-transfected into FreeStyle™ 293-F cells, respectively. The BsAbs CP.PSMA x EGFR and KIH.PSMA x EGFR were expressed and purified as described in method 2.2 (Fig. 1B-C). The anti-gp120 (HIV) Fab arm and anti-gp41 (HIV) sdAb were used to generate bispecific control antibodies by cFAE (Burton et al., 1994) (Hulsik et al., 2013) (Fig. 1D).

3.2. Purity evaluation of BsAbs

In order to determine the purity of Fab x sdAb-Fc format BsAbs, we performed Capillary Electrophoresis Sodium Dodecyl Sulfate (CE-SDS) analysis. Under non-reducing conditions, the BsAbs generated by cFAE, CP and KIH showed clear separation from parental antibodies with a 95.9%, 94.6% and 88.9% purity for DB.PSMA x EGFR, CP.PSMA x EGFR or KIH.PSMA x EGFR, respectively. (Fig. 2A-C). The purity of three bispecific control antibodies DB.PSMA x gp41, DB.gp120 x EGFR, and DB.gp120 x gp41 was also evaluated by CE-SDS showing 94.8%, 94.2%, and 94.5% purity, respectively (data not shown). These data demonstrate that Fab x sdAb-Fc format BsAbs can be generated efficiently, and with high quality, using various heavy chain heterodimerization strategies. The BsAb KIH.PSMA x EGFR, which was produced with a relative low purity (88.9%), was produced by KIH using mutations T366W in the “knob” heavy chain and T366S, L368A, Y407V in the “hole” heavy chain (Fig. 1C). By introducing two additional mutations (S354C in the “knob” heavy chain and Y349C in the “hole” heavy chain) such a construct is expected to further increase the efficiency of heterodimerization and thus improve the purity of this BsAb construct (Merchant et al., 1998).

Other than hydrophobic interaction chromatography (HIC), Cation exchange chromatography (CIEX), Liquid chromatography–mass spectrometry (LC-MS), and CE-SDS, SDS-PAGE is a convenient and low demand in equipment method to assess the successful formation and estimate the purity of the Fab x sdAb-Fc BsAb format. Thus, SDS-PAGE analysis of each purified BsAb was performed. Under non-reducing conditions, the desired BsAbs are expected to show a predominant band with a MW of ~127 kDa, whereas parental antibodies should show predominant bands with a MW of ~93 kDa or ~ 163 kDa, respectively (Fig. 2D). For BsAb CP.PSMA x EGFR and BsAb KIH.PSMA x EGFR,

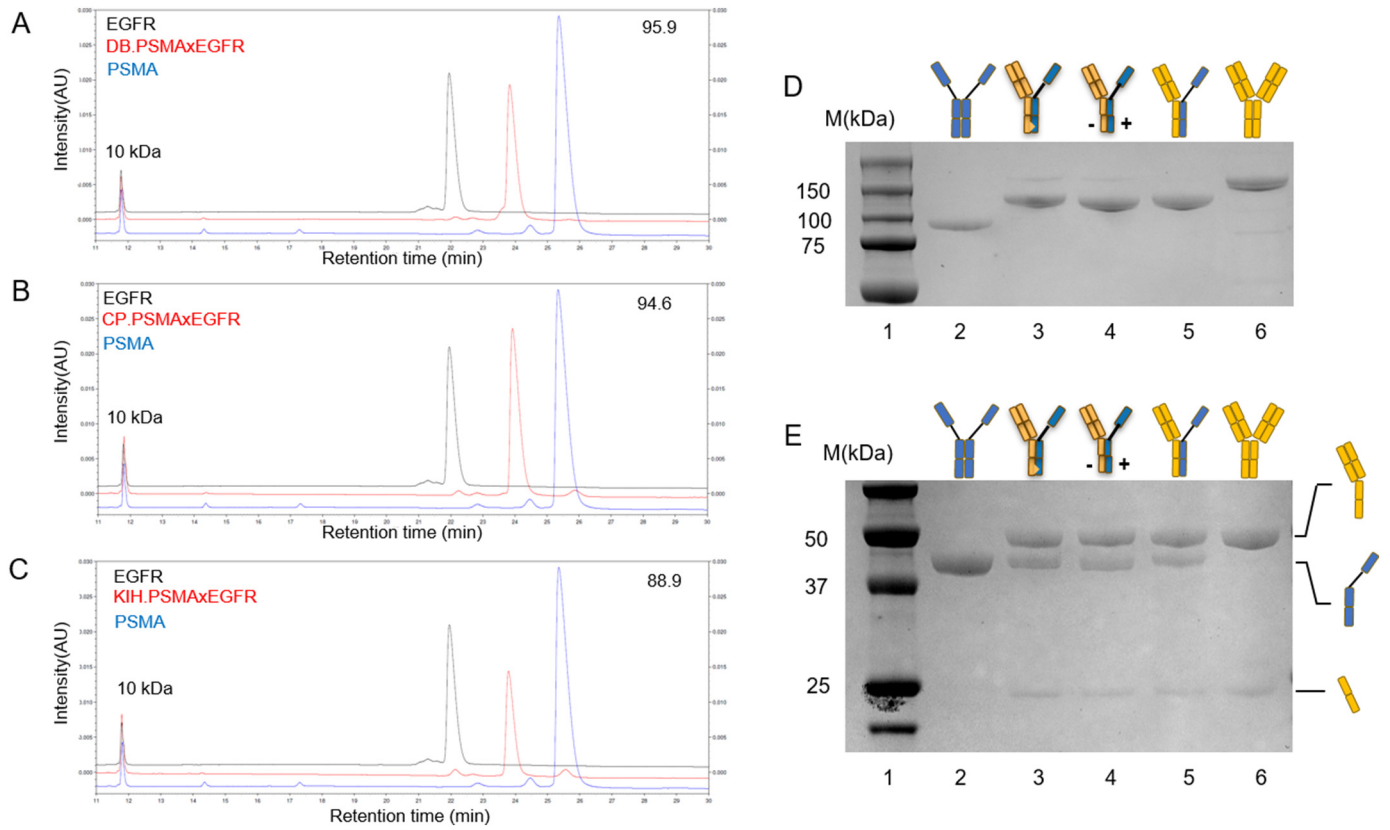


Fig. 2. CE-SDS and SDS-PAGE analysis for the various Fab x sdAb-Fc BsAb formats. (A-C) Purity of (A) DB.PSMA x EGFR, (B) CP.PSMA x EGFR and (C) KIH.PSMA x EGFR evaluated by CE-SDS. 10 kDa standard marker was used for the calibration of retention time for each trace. Numbers represent percentage of BsAb product (middle red peaks). The peaks of parental antibody were shown in black(EGFR) or blue(PSMA) respectively; (D-E) Detection and separation of BsAbs and parental antibodies by coomassie blue staining of SDS-PAGE under (D) non-reducing or (E) reducing condition. Lane 1, MW ladder; lane 2, EGFR HcAb; lane 3, KIH.PSMA x EGFR; lane 4, CV.PSMA x EGFR; lane 5, DB.PSMA x EGFR; lane 6, PSMA mAb. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

additional minor bands were detected at a size of ~160 kDa, which indicated a minor contamination of the parental PSMA mAb in this batch. Under reducing conditions, one band for EGFR HcAb, two bands for PSMA mAb and three bands for BsAbs were detected, as expected (Fig. 2E). The bands at ~50 kDa detected in BsAbs and PSMA mAb represent the heavy chain of the PSMA mAb, while the MW bands at ~46 kDa detected in EGFR HcAb and BsAbs represent the heavy chain of the EGFR HcAb. The bands at ~25 kDa detected in BsAbs and PSMA mAb represent the light chain of the PSMA mAb.

Taken together, these data indicate that BsAbs can be efficiently generated with high purity. Additionally, due to the MW difference between BsAbs and parental antibodies, this format of BsAb can be readily further purified from undesired by-products based on MW by preparative Size Exclusion Chromatography.

3.3. Monomericity evaluation of BsAbs

To further analyse the aggregation and degradation level of BsAbs in our purified batches, we performed analytical Size Exclusion Chromatography (HP-SEC). After HP-SEC gel filtration, a predominant peak with an area of 99% for BsAb DB.PSMA x EGFR, 98.5% for BsAb CP.PSMA x EGFR and 98.4% for BsAb KIH.PSMA x EGFR was detected (Supplementary Fig. 1). The aggregation and degradation level of three bispecific control antibodies DB.PSMA x gp41, DB.gp120 x EGFR, and DB.gp120 x gp41 were also evaluated by HP-SEC which showed 98.8%, 98.8%, and 99% monomericity, respectively (data not shown).

3.4. BsAbs analysis for isoelectric point

Generated BsAbs and parental antibodies were further analysed by Capillary Isoelectric Focusing cIEF (Fig. 3). The BsAbs and parental antibodies showed clearly different isoelectric points (pI). We observed that the peaks of BsAbs were not detected precisely in the middle of the peaks of two parental antibodies but were closer to the Sam103 peaks. The peak pattern in this novel BsAb format can be explained by the different mass contribution of each parental antibodies (~163 kDa versus ~93 kDa).

3.5. Binding activity of BsAbs

To test the binding activity of each arm of the BsAbs, stably transfected CHO/mEGFR and CHO/mPSMA were generated and used for binding assays. Binding activity of BsAb DB.PSMA x EGFR and parental antibodies specific for mEGFR or mPSMA were determined by flow cytometry, using unlabelled primary antibodies followed by FITC-labelled secondary antibody for detection. BsAb DB.PSMA x EGFR showed dose-dependent binding to both antigens. Monovalent binding activity of BsAb DB.PSMA x EGFR to CHO/mEGFR cells showed relative similar potency to bivalent binding by EGFR HcAb (EC50 0.87 nM versus 0.28 nM) (Fig. 4A-B). For mPSMA binding, BsAb DB.PSMA x EGFR showed strong reduction in binding to CHO/mPSMA compared to the parental bivalent PSMA mAb (EC50 219 nM versus 0.32 nM) (Fig. 4C-D). These data demonstrate that each antibody retained its specificity within the bi-specific construct. In addition, and in contrast to the EGFR specific sdAb RR359, the Sam103 PSMA antibody depends on avidity for high affinity binding to its target.

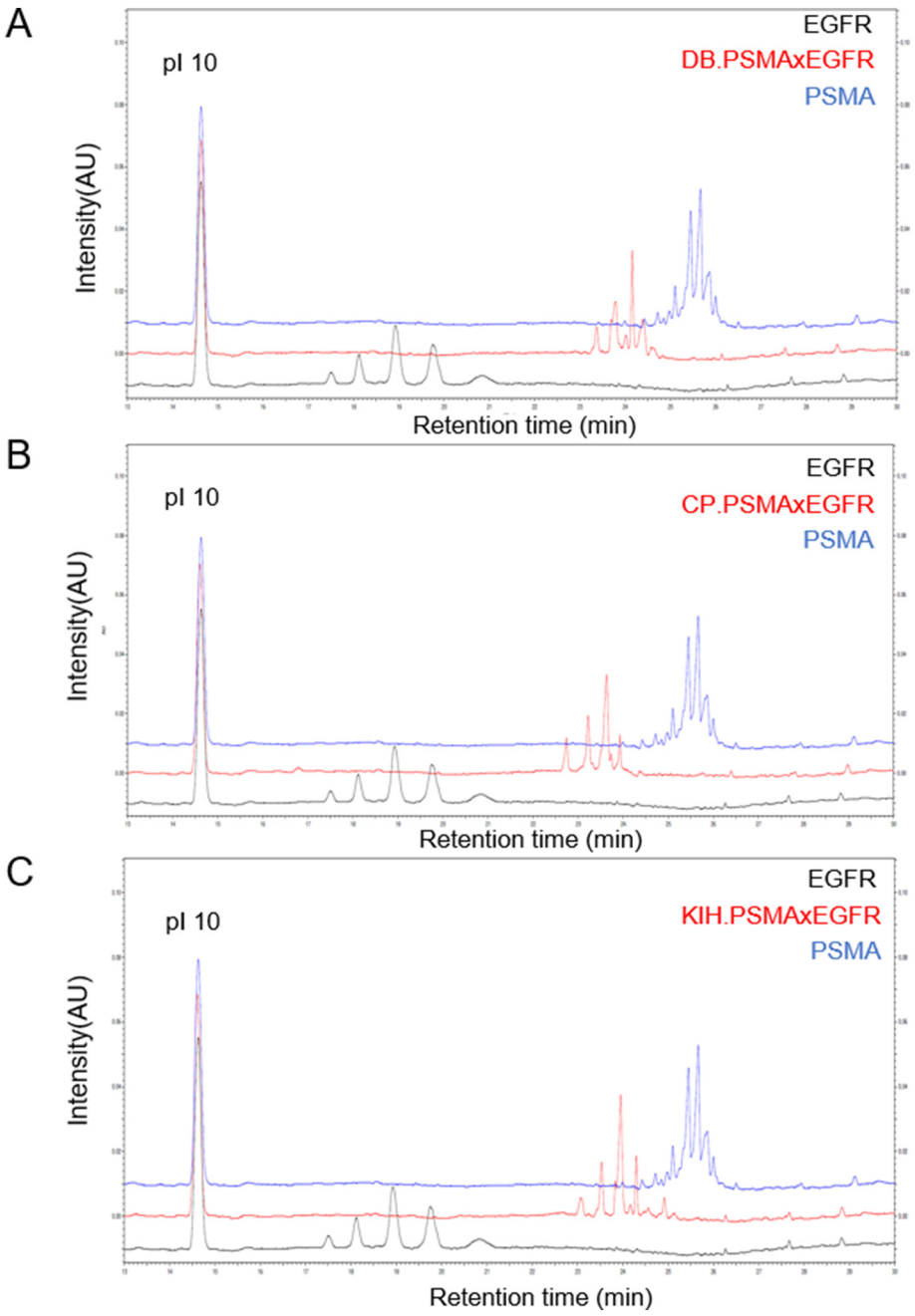


Fig. 3. cIEF analysis for the Fab x sdAb-Fc BsAbs. (A) DB.PSMA x EGFR, (B) CP.PSMA x EGFR (C) KIH.PSMA x EGFR and parental antibodies. A gel filtration standard (protein pI 10) was included for the calibration of retention time for each trace. The BsAbs are shown in red (middle line in each diagram). The parental antibodies are shown in blue for anti-PSMA mAb (top line in each diagram) and black for anti-EGFR HcAb (bottom line in each diagram), respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Remarkably, a qualitative examination of the binding curves revealed a differential staining plateau for the two EGFR-specific antibody constructs. At a saturated concentration, BsAb DB.PSMA x EGFR binding to CHO/mEGFR cells plateaued at an MFI of ~1250, which was approximately 2 times as high as the plateau value for EGFR HcAb, which had an MFI of ~750 (Fig. 4A). The differences between MFI plateau reached by each antibody construct might be due to the difference between monovalent and bivalent binding, to be definitively confirmed using monovalent parental controls. In case of monovalent binding, one Ab construct binds one receptor. However, in case of bivalent binding one Ab construct is capable of binding two receptors resulting in a lower absolute amount of cell bound antibody. Correspondingly, in this experimental set-up, the higher staining plateau for BsAb compared to HcAb is likely the result of pure monovalent binding by the BsAb versus substantial bivalent binding for HcAb.

Combined, these data demonstrate that each arm of BsAb DB.PSMA x EGFR maintained the binding activity to its corresponding antigen.

3.6. Simultaneous binding of BsAb

To determine whether our bi-specific constructs retained their capacity to bind to both antigens simultaneously, we performed bio-layer interferometry (BLI), using an Octet machine, and physical cell-bridging experiments, using FACS analysis. The binding characteristics of the BsAb DB.PSMA x EGFR and three bispecific control antibodies to recombinant mEGFR-His and mPSMA-His were analysed using Octet. In

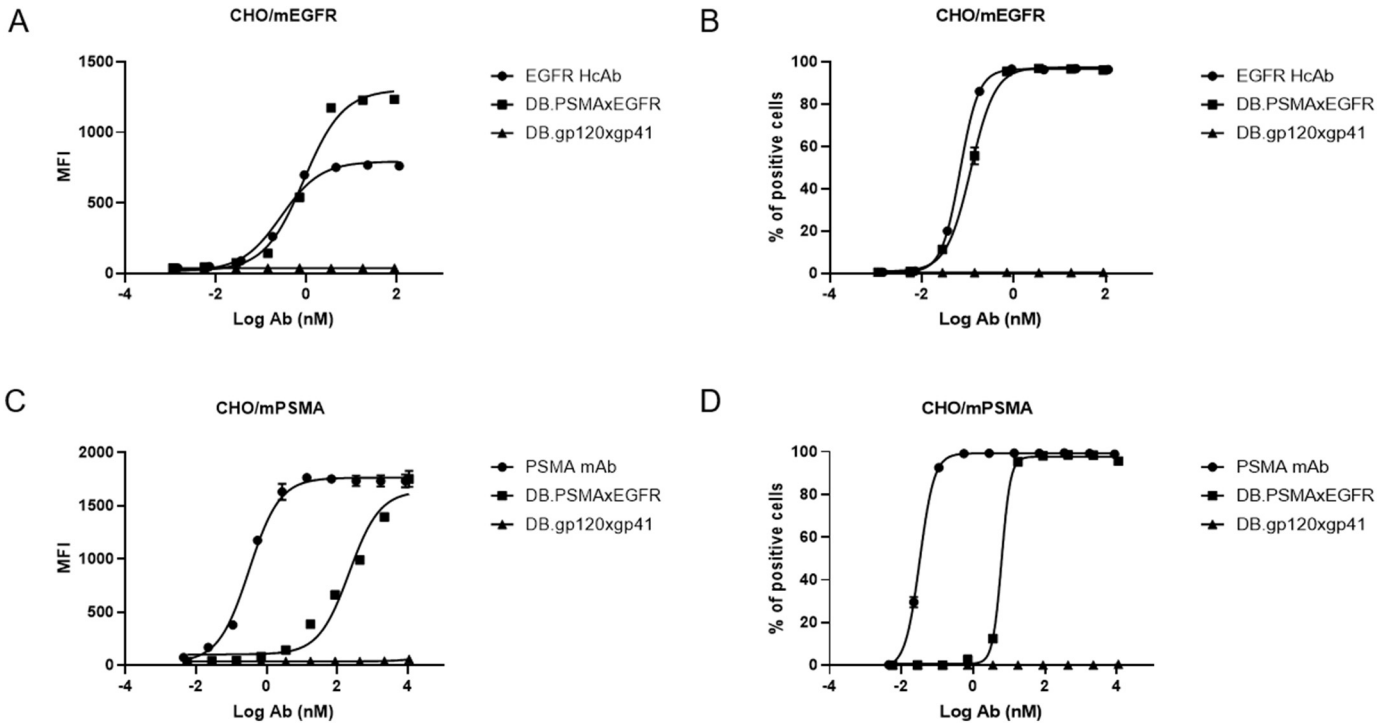
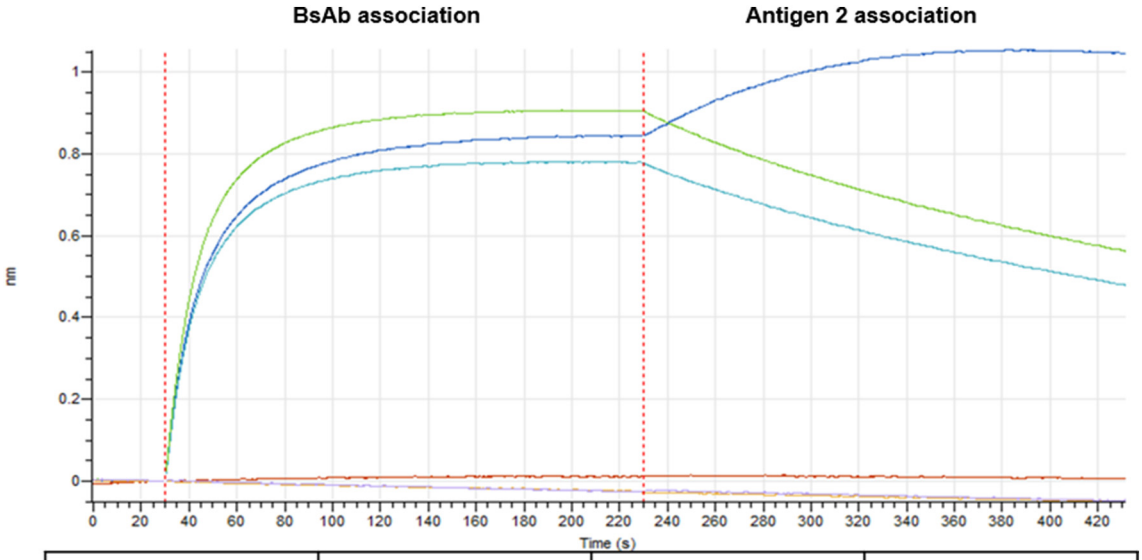


Fig. 4. Flow cytometry analysis of BsAbs and parental antibodies binding on CHO/mEGFR or CHO/mPSMA cells in a dose-dependent fashion. (A-B) CHO/mEGFR cells stained by parental anti-EGFR HcAb, DB.PSMA x EGFR or control antibody DB.gp120 x gp41 respectively. (C-D) CHO/mPSMA cells stained by parental anti-PSMA mAb, DB.PSMA x EGFR or control antibody DB.gp120 x gp41 respectively. Each data point is the mean ± SD of triplicates.



Curve	Antigen 1 loading	BsAb	Antigen 2 association
Blue	mEGFR-HIS	PSMA x EGFR	mPSMA-HIS
Turquoise	mEGFR-HIS	PSMA x EGFR	-
Green	mEGFR-HIS	gp120 x EGFR	mPSMA-HIS
Yellow	mEGFR-HIS	PSMA x gp41	mPSMA-HIS
Purple	mEGFR-HIS	gp120 x gp41	mPSMA-HIS
Red	-	PSMA x EGFR	mPSMA-HIS

Fig. 5. BLI analysis of BsAb DB.PSMA x EGFR and BsAb controls for simultaneous binding to recombinant mEGFR and recombinant mPSMA. The BsAb association to mEGFR loaded biosensors is displayed, followed by the association of mPSMA to the BsAb-EGFR complex for the various BsAb antibodies as indicated in the table.

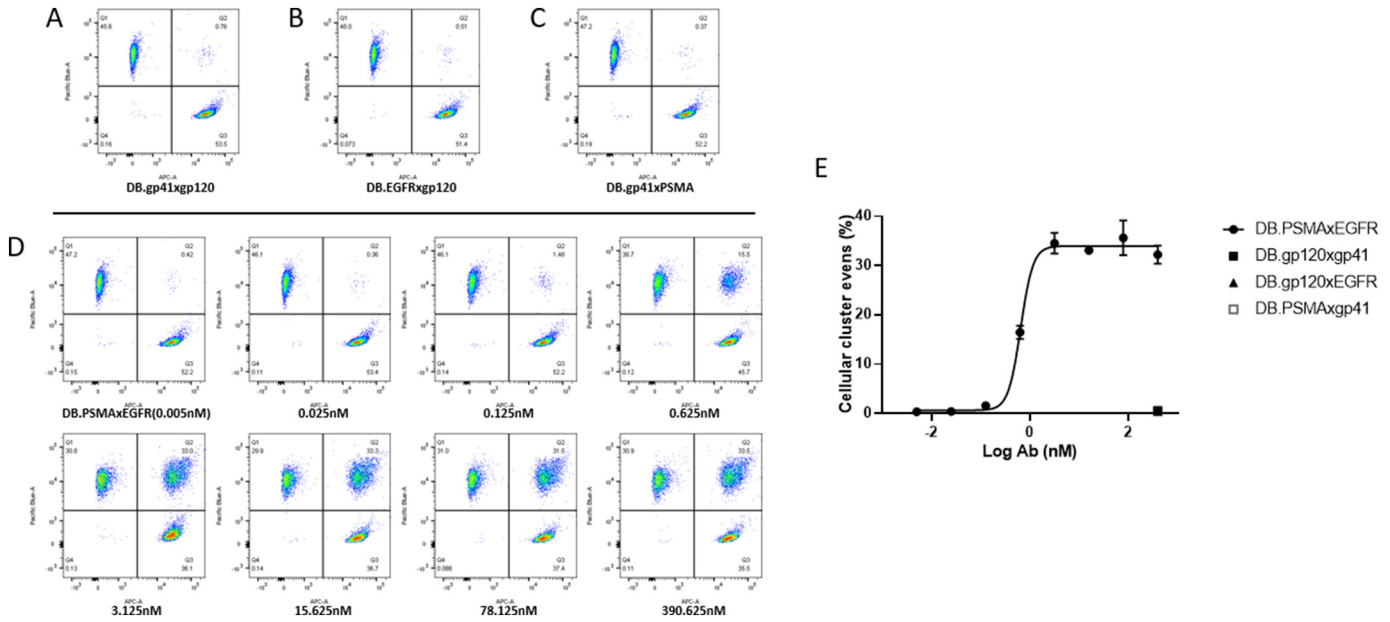


Fig. 6. Flow cytometry analysis of BsAbs inducing cellular clustering by simultaneous binding to CHO/mEGFR and CHO/mPSMA cells. (A-C) 1:1 mixed CHO/mEGFR and CHO/mPSMA cells incubated with 390.63 nM bispecific control antibodies; (D) 1:1 mixed CHO/mEGFR and CHO/mPSMA cells incubated with increasing concentration of DB.PSMA x EGFR. (E) Quantification of flow cytometry results by displaying the percentage of events that represents cellular clusters. Means ± SD of a representative experiment (n = 3) performed in triplicates are shown.

short, mEGFR recombinant protein was loaded on the biosensor, followed by incubation of the various BsAbs to allow mEGFR specific binding. Thereafter, the biosensors were incubated with mPSMA recombinant protein to assess binding of mPSMA to the mEGFR-BsAb complex. As shown in Fig. 5, BsAb DB.PSMA x EGFR was able to bind both antigens, recombinant mEGFR and mPSMA, at the same time. Bispecific control DB.gp120 x EGFR showed only binding to mEGFR, while bispecific control DB.PSMA x gp41 showed no binding activity towards the mEGFR loaded biosensor (Fig. 5 and Supplementary Fig. 2).

In addition, a cell bridging experiment was designed to test the ability of BsAb DB.PSMA x EGFR and three bispecific control antibodies to simultaneously bind to CHO/mEGFR and CHO/mPSMA cells. CHO/mEGFR and CHO/mPSMA cells were stained with two different cell staining dyes, subsequently mixed together at a 1:1 ratio, and incubated with BsAb (DB.PSMA x EGFR) or control antibodies. Flow cytometry results showed that only BsAb DB.PSMA x EGFR could serve as a bridge binding CHO/mEGFR and CHO/mPSMA cells together to form cell clusters in a dose dependent fashion leveling out at ~30% cell clusters (Fig. 6). Although we quantified ~30% cellular cluster events, the number of clustered cells is likely higher than 30% of total cells counted because the flow cytometry set up did not enable distinguishing between doublets, triplets, or higher order cell clusters. Clustering by BsAb is reported to be a direct function of absolute number of target antigens expressed on each cell (Lopez-Albaitero et al., 2017; Oberst et al., 2014; Laszlo et al., 2014) and although we detected ~20,000 target molecules on average for mPSMA and mEGFR on the respective transfected clones (data not shown), a significant number of either clonal population would display a number of antigens below the clustering threshold. Moreover, stability of clustering is also determined by fluid shear force leading to disruption of weakly formed clusters before they pass the detector. Hence, a maximum of 100% cell clusters was not expected.

Combined, these data show that the BsAb DB.PSMA x EGFR is capable to simultaneously bind to its two antigens.

4. Discussion

Although the concept of BsAb has been used for a very long time, the production and purification steps during BsAb development remain one of the major challenges for their application to research and clinical development. Specifically, heavy-light chain mis-pairing has remained one of the major challenges to obtain pure BsAb. Approaches using a common light chain, present a challenge when making use of already pre-existing and validated antibodies (Merchant et al., 1998). Furthermore, approaches which introduce mutations at the contact points of VH/VL or CH1/CL sometimes compromise the stability of the BsAb (Atwell et al., 1997); while approaches that exchange the VH-VL or the CH1-CL domains by domain crossover between the heavy and light chain Fab domains can even damage antigen binding ability (Masuda et al., 2006) (Chailyan et al., 2011) (Herold et al., 2017). All these approaches require additional protein engineering and redetermination of the physicochemical and biological characteristics of engineered BsAb, making a standard mass production of novel bi-specific antibodies challenging.

In the present study, we describe a novel approach to overcome heavy-light chain mis-pairing, by establishing the Fab x sdAb-Fc format of BsAb composed of a conventional antigen-binding fragment (Fab), a single domain antibody (sdAb) and a mouse IgG2a Fc. We demonstrated that the Fab x sdAb-Fc BsAb format can functionally be expressed and assembled in a single FreeStyle™ 293-F host, using KIH and CP dimerization strategies, or formed artificially by cFAE; reaching high purity and retaining their capacity to bind both their target antigens simultaneously.

This format of BsAb also provides additional advantages for further purification. As previously described, the Fab x sdAb-Fc format BsAbs showed ~35 kDa MW difference from each parental antibody which is sufficient for further separation based on size exclusion chromatography (SEC). Affinity chromatography might be another consideration to further purify this format of BsAb. Affinity chromatography is a type of chromatographic method used for purifying biological molecules within a mixture based on highly specific biological interactions between two molecules, for instance, interactions between antibody and antigen (Urh et al., 2009). Next, for the Fab x sdAb-Fc format, CH1

selective chromatography can help to remove HcAbs. An IgG CH1 domain specific antibody could be conjugated to a resin to capture BsAb and the parental antibody with CH1 domain, while HcAb can not bind, in this way further improving the purity of BsAb.

In the presence study, the hinge between the sdAb and Fc was designed to mimic the length of an entire CH1 domain, thus extending the length of the sdAb arm similar to that of conventional Fab. However, the hinge of the HcAb can be designed various for different applications. T cell-redirecting BsAbs with Fc might benefit from a short hinge design. The distance between tumour cells and effector T cells has been demonstrated essential for T cell mediated tumour cell killing (Bluemel et al., 2010). A shorter distance between two arms can redirect T cells closer to tumour cells which might lead to better tumour cell elimination. For targeting of tumour associated antigens the hinge of Fab x sdAb-Fc BsAb can be optimized based on the distribution, density and extracellular size of antigens with special attention to the distance between two antigens expressed on the tumour cell surface. Nevertheless, how different hinges affect the functionality, stability and flexibility of this format of BsAb still needs to be investigated further.

Taken together, we describe here a novel approach of constructing bi-specific antibodies, which has a number of different advantages over traditional approaches. It is foreseeable that the potential applications for this format will only grow as the field of BsAb application in academic and clinical settings continues to evolve.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2020.112811.

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