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PSMA-targeted bispecific Fab conjugates that engage T cells

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ABSTRACT

Bioconjugate formats provide alternative strategies for antigen targeting with bispecific antibodies. Here, PSMA-targeted Fab conjugates were generated using different bispecific formats. Interchain disulfide bridging of an αCD3 Fab enabled installation of either the PSMA-targeting small molecule DUPA (SynFab) or the attachment of an αPSMA Fab (BisFab) by covalent linkage. Optimization of the reducing conditions was critical for selective interchain disulfide reduction and good bioconjugate yield. Activity of αPSMA/CD3 Fab conjugates was tested by *in vitro* cytotoxicity assays using prostate cancer cell lines. Both bispecific formats demonstrated excellent potency and antigen selectivity.

Bispecific antibodies (BsAbs) that engage the immune system have enormous potential as cancer therapeutics.[1] Such BsAbs confer dual specificities for distinct antigens or epitopes using a single macromolecule. Hence, the ability of BsAbs to support protein complex formation can be exploited to bring two cell types in close proximity. Simultaneous engagement of a tumor antigen and a surface receptor on an effector cell can lead to tumor cell killing. Monocytes, macrophages, natural killer cells, and T cells have been successfully redirected by BsAbs towards tumors.[2]

In particular, cytotoxic T cells are highly potent killers that can infiltrate tumors, undergo serial lysis, and proliferate after stimulation.[3] The approvals of αEpCAM/CD3 BsAb catumaxomab (Removab®) and αCD19/CD3 bispecific T cell engager (BiTE) blinatumomab (Blincyto®) have demonstrated the remarkable promise of this approach.[4] Many clinical programs based on the recruitment of cytotoxic T cells to tumors have also shown encouraging results.[5] While one arm of the BsAb binds CD3 of the T cell receptor complex, the second arm binds a tumor-associated antigen. Thus, the BsAb format is critical for enabling productive immunological synapse formation leading to T cell activation and tumor killing.

Initial efforts to generate BsAbs relied on hybrid hybridoma or chemical crosslinking of F(ab′) fragments to yield covalent $F(ab')_2$ molecules (BisFabs).[6] However, recombinant methods have facilitated the development of engineered antibodies and antibody fragments. Validated formats such as knob-into-holes[7,8] and DuoBody[9] rely on IgG heavy chain dimerization interface engineering, whereas BiTE[10] and dual-affinity re-targeting (DART)[11] platforms are based on tandem single-chain Fv (scFv) constructs and diabody-like fragment formats, respectively. Nonetheless, chain mispairing has been problematic in optimizing full-length BsAb formats.[5,12] Identification of stable antibody fragments with good biophysical properties has similarly been a limitation.[12,13] Expression and purification can, in both cases, become an obstacle to reaching the development stage. Hence, exploration of novel bispecific formats is warranted.

Semisynthetic approaches for BsAb production may overcome some of these limitations imposed by recombinant methods.[14,15] Production of the two binding components separately followed by covalent attachment can potentially avoid problems like incorrect domain association and protein misfolding that sometimes occur in engineered formats. Nonetheless, early efforts developing chemically generated BsAbs were prone to their own shortcomings, such as low yield and product heterogeneity.[16] Improvements in linker design and bioconjugation processes have greatly advanced the field in recent years.[16,17] For example, optimized reduction/re-oxidation of F(ab′) fragments prior to conjugation decreased homodimer formation and aggregate species during BisFab preparation.[18,19] Similarly, we recently improved the yield and homogeneity of chemically linked IgG2 BsAbs by optimizing the reduction step prior to disulfide bridging and orthogonal linkage.[20] We have extended this disulfide bridging strategy to the modification of Fab domains, allowing production of multiple bispecific formats.

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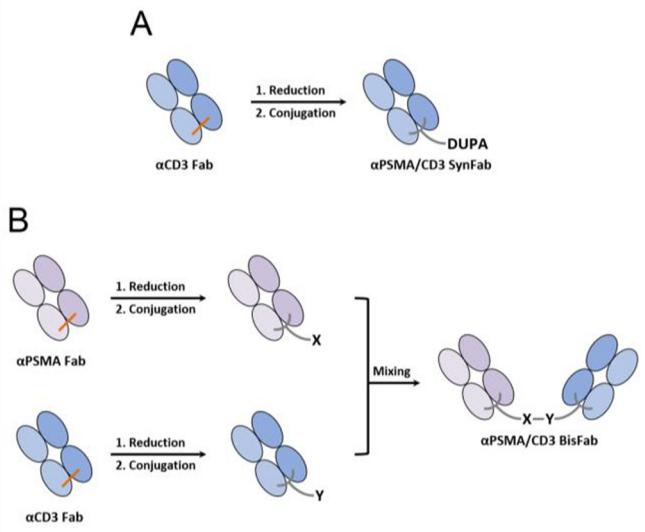
αPSMA/CD3 Fab conjugates were prepared using either synthetic (SynFab) or Fab (BisFab) targeting modalities for the redirection of T cells to prostate tumors. To this end, selective reduction of the αCD3 Fab interchain disulfide bond was performed then followed by installation of either αPSMA small molecule or Fab targeting moiety (Scheme 1). The small molecule 2-[3-(1,3-dicarboxypropyl)ureido]pentanedioic acid (DUPA)[21,22] was directly conjugated to αCD3 Fab using dibromomaleimide (DBM)[23] linker **1** (Fig. 1, Schemes S1 and S2). BisFab was readily formed once orthogonal azide and dibenzylcyclooctyne (DBCO) groups were introduced by DBM linkers **2** and **3**, respectively, to Fabs enabling chemical linkage by copper-free click chemistry (Fig. 1 and Scheme S3).



Scheme 1. Synthetic route to Fab conjugates by disulfide bridging. (A) Direct modification of αCD3 Fab using a linker containing the DUPA targeting modality. (B) Fab conjugation with linkers bearing azide (X) or DBCO (Y) orthogonal groups for copper-free click chemistry.

Fab proteins were prepared by enzymatic digestion of IgG1 antibodies using the His-tagged SpeB cysteine protease FabULOUS® (Fig. S1). Conditions were adapted for overnight digestion that were directly compatible with subsequent chromatography. Purification was performed by sequential Ni Sepharose and Protein A column chromatography steps to remove enzyme and any Fc-bearing proteins, respectively. Both αPSMA and αCD3 Fabs were recovered with good purity and no residual full-length antibody or partially digested products as evidenced by analytical hydrophobic interaction chromatography (HIC) HPLC (Fig. S2). However, a preparative HIC FPLC polishing step further improved Fab homogeneity (Fig. S3). To ensure Fab purity, αCD3 Fab was compared to αCD3 IgG1 in an *in vitro* cytotoxicity assay using human peripheral blood mononuclear cells (PBMCs) to evaluate nonspecific T activation and subsequent T cell-mediated lysis of LNCaP cells (Fig. S4). No killing was observed with αCD3 Fab neither with material after the initial purification nor after additional HIC polishing.

Next, Fab reduction was evaluated to identify the preferred conditions for selective interchain disulfide modification. We previously observed more selective reduction of IgG2 hinge disulfide bonds using dithiothreitol (DTT) over tris(2-carboxyethyl)phosphine (TCEP) as it is a milder reducing agent.[20] Therefore, we anticipated that selective reduction of Fab interchain disulfide bonds could also be achieved with DTT. αCD3 Fab was reduced with 10, 20, or 30 equivalents of DTT for 1 h at room temperature. Samples were then passed through a Sephadex G-25 column to remove excess reducing agent, conjugated with 10 equivalents DBM-PEG8-DBCO linker overnight at room temperature (increasing equivalents of DBM linker did not alter conjugate yield, data not shown), and remaining linker was removed by centrifugal filtration.

Analytical HIC analysis of Fab conjugation reactions showed increased conversion to product with higher equivalents of DTT (Fig. 2). Maximal conversion was observed with the 20 and 30 equivalents DTT conditions, which typically ranged from 70 to 85% over multiple reactions. Interchain crosslinking was confirmed by SDS-PAGE under reducing conditions as conjugated Fab maintained its ~50 kDa molecular weight while parental Fab was reduced to ~25 kDa, representing each heavy and light chain

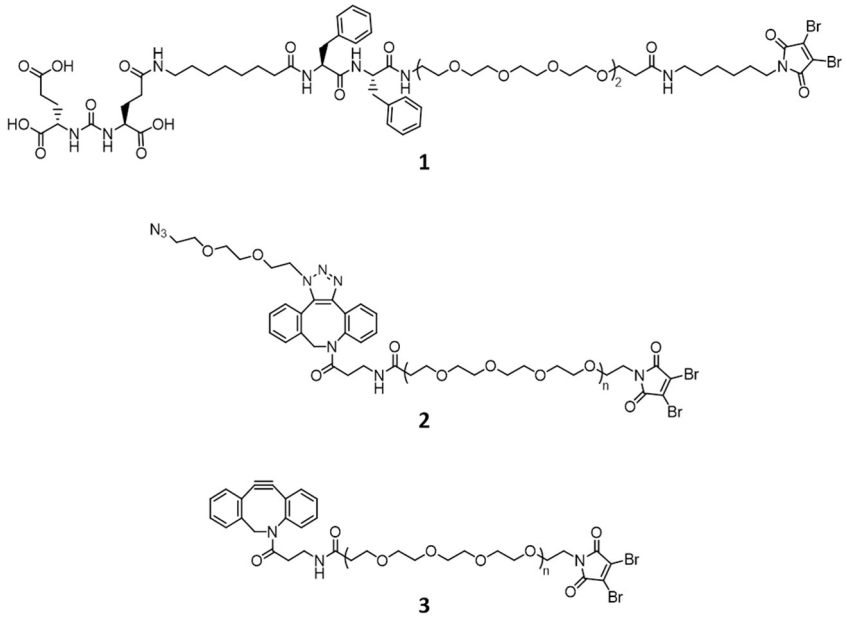


Fig. 1. Structures of dibromomaleimide linkers used for preparation of SynFab (**1**) and BisFab (**2**–**3**) constructs, n = 1 or 2.