

## PREPARATION OF BISPECIFIC ANTIBODY MIMETICS

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**Background:** Fab-PEG-Fab (FpF) molecules have been shown to act as IgG mimetics [1]. Each antibody Fab is conjugated at both termini of a *di-bis*-alkylation PEG reagent site-specifically by disulfide bridging conjugation. We now wish to examine the binding properties of bispecific FpFs, which would be derived from two different Fabs and are designed to act as bispecific antibody mimetics. The aim of this study was to compare the preparation of bispecific FpFs using the *di-bis*-alkylation reagent and *mono-bis*-alkylation reagents functionalized on the other PEG terminus with a functional group capable of undergoing a copper free 1,3-dipolar cycloaddition (click reaction) between an azide (N<sub>3</sub>) and the strained alkyne in dibenzocyclooctyne (DBCO) [2].

**Methods:** *Synthesis of mono-bis-alkylation PEG "click" reagents:* BocNH-PEG-NH<sub>2</sub> (5 or 10 kDa) was first functionalised with the *bis-alkylation* conjugation moiety using isobutyl 1,2-dihydro-2-isobutoxy-1-quinolinecarboxylate (IIDQ) in acetonitrile and the product purified by precipitation in cold acetone (dry-ice). The Boc was then removed using 30% TFA in dichloromethane, and following roto-evaporation, the TFA-amine salt was precipitated in cold acetone. The second PEG-amino terminus was then functionalised with a DBCO-NHS analogue using 4-dimethylaminopyridine (DMAP) in acetonitrile. The final *mono-bis*-alkylation-PEG-DBCO reagent was isolated after precipitation in cold acetone. The azide partner was synthesised by functionalising NH<sub>2</sub>-PEG-N<sub>3</sub> with the *bis-alkylation* conjugation moiety using IIDQ in acetonitrile and isolated after precipitation in cold acetone.

**Results:** Analysis of H-NMR spectra indicated that the amide coupling reactions occurred in high efficiency for both the *mono-bis*-alkylation PEG DBCO and N<sub>3</sub> reagents with final reagent purities > 90% based on analysis of integrals of the diagnostic peaks due to the functional groups on each termini and the PEG methylenes. Fab conjugation at the *bis*-alkylation moiety for each reagent proceeded in the same manner as observed previously for Fab PEGylation [3]. The Fab-PEG<sub>5k</sub>-DBCO and Fab-PEG<sub>10k</sub>-N<sub>3</sub> conjugates were then mixed in phosphate buffered saline for 24 hours, SDS-PAGE indicated the formation of the click product at approximately 120 kDa. Purification by ion exchange chromatography yielded the bispecific FpF. Use of the *di-bis*-alkylation reagent described in [1] and sequential additions of the two Fabs also yielded the desired bispecific FpFs, but the reaction mixture was more difficult to purify.

**Conclusions:** The *mono-bis*-alkylation PEG-DBCO and PEG-N<sub>3</sub> reagents allow for a simpler preparation of bispecific FpFs than bispecific FpFs prepared using a *di-bis*-alkylation PEG reagent.

### References:

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