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| **Evaluating the protein stability of the click bispecific antibody**  |
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| **Background:** Bispecific antibodies have emerged as a rapidly growing class of biologics with the potential to transform the field of biotherapeutics by providing a novel approach for targeted therapy and treatment of a wide range of diseases. Bispecific antibodies are capable of binding two different targets simultaneously and have been successfully used in oncology (blinatumomab and emicizumab) and ocular neovascularisation (faricimab). While they can be engineered using recombinant methods, chemical conjugation has emerged as a promising approach. One chemical approach is to use bis-alkylating polyethylene glycol (PEG) to conjugate with two independently produced antibody fragments (Fab) to create PEG-Fab products. The PEG-Fabs can further covalently bond together through the inverse electron requirement of the Diels-Alder “click” reaction to produce bispecific Fab1-PEG-Fab2 molecules. Protein stability is the major concern in the development of bispecific antibodies, particularly with regards to local delivery over prolonged periods of time. Light and heavy chain dissociation in Fabs and detachment of PEG from bispecific conjugates are the main instability issue. Therefore it is important to determine whether the stability of proteins are conserved in the liquid state of the bispecific Fab1-PEG-Fab2. In this study, we aim to prepare Fab1-PEG-Fab2 using click bis-alkylating PEG to target ocular inflammation disease and examine its storage stability in its liquid state. |
| **Methods:** Fabs were obtained using proteolytic digestion of IgGs using both soluble and immobilised papain. To digest 100 mg of IgG,15 mg of soluble papain was added to a digestion solution containing 80 mM L-cysteine at pH 7.0 for 40 minutes at 37 degrees. Fab fragments were then purified using protein L column and size exclusion chromatography columns. The purity of obtained Fabs was studied using SDS-PAGE analysis. To prepare bispecific Fab1-PEG-Fab2, in two different containers, Fab1 and Fab2 (targeting two different antigens) have incubated with DTT reducing agent to open up accessible disulfide bonds necessary for bis-alkylating reaction with PEG-Bis alkylating agents. X-PEG-Fab1 and Y-PEG-Fab2 were separately purified from un-reacted PEG and Fabs using ion-exchange chromatography and then click together at pH 7.8 to prepare bispecific Fab1-PEG-Fab2. |
| **Results:** Large-scale IgG digestion resulted in the production of 28.5 mg of pure Fab from 100 mg of IgG. For better yield, higher concentration of L-cysteine (80 mM vs. 50 mM) and a longer digestion time (40 minutes vs. 30 minutes) was used. As a result, a greater quantity of pure Fab fragments was obtained after complete digestion, making the purifying process simpler. Obtained Fabs was then reacted with DTT and then incubated with x-PEG-bis-alkylating reagent to produce Fab1-PEG-X and Fab2-PEG-Y. Purification using ion-exchange chromatography, resulted in production of pure mono-PEG Fabs. Bispecific conjugate was then obtained by the click reaction between X-Y moieties in the combined Fab1-PEG-X and Fab2-PEG-Y at pH 7.8. Investigation of the stability of bispecific in liquid form, Fab1-PEG-Fab2, shown that there was no PEG detachment or light or heavy chains dissociation after 12 weeks at 4 degrees and 4 weeks at 37 degrees. |
| **Conclusions:** We have prepared bispecific antibodies, Fab1-PEG-Fab2, to bind two ocular inflammatory targets using bis-alkylation “click” chemical conjugation methods. The Fab1-PEG-Fab2 bispecific has kept its storage stability in liquid form, but the next step is to investigate the stability of a newly produced click bispecific in solid-state form after being lyophilized in the hopes of producing stable solid material for depot fabrication. The development of a stable biologic for treatment of ocular inflammation has remained elusive. Hence, preparation of stable bispecific Fab1-PEG-Fab2 could potentially revolutionise its treatment.  |