Effect of PEGylation on the Solution Conformation of Antibody Fragments

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ABSTRACT: Covalent attachment of poly(ethylene glycol) (PEG) to therapeutic antibody fragments has been found effective in prolonging the half-life of the protein molecule *in vivo*. In this study analytical ultracentrifugation (AUC) in combination with small angle X-ray scattering (SAXS) has been applied to a number of antibody fragments and to their respective PEGylated conjugates. Despite the large increase in molecular weight due to the attachment of a 20–40 kDa PEG moiety, the PEGylated conjugates have smaller sedimentation coefficients, s, than their parent antibody fragments, due to a significant increase in frictional ratio f/f_o (from ~1.3 to 2.3–2.8): the solution hydrodynamic properties of the conjugates are clearly dominated by the PEG moiety (f/f_o ~3.0). This observation is reinforced by SAXS data at high values of r (separation of scattering centres within a particle) that appear dominated by the PEG part of the complex. By contrast, SAXS data at low values of r suggest that there are no significant conformational changes of the protein moiety itself after PEGylation The location of the PEGylation site within the conjugate was identified, and found to be consistent with expectation from the conjugation chemistry. © 2007 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 97:2062–2079, 2008

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INTRODUCTION

Biomedical exploitation of the discrimination and affinity of antibody binding properties has been advanced by biotechnological developments. There are now 18 commercially available monoclonal antibody products and more than 100 in clinical development. In the near future, engineered antibodies are predicted to account for >30% of all revenues in the biotechnology market.[1] High-level expression systems have been developed for mammalian cell expression of antibodies; however, the large quantities needed in therapeutic doses make them prohibitively costly. Antibody fragments (Fab', Fv and scFv)[2–4] and engineered variants (diabodies, triabodies, minibodies and single-domain antibodies)[1,5–7] that can be readily produced in large-scale in microbial systems are now emerging as credible alternatives for *in vitro* immunoassays and *in vivo* tumour-targeting therapy.[8]

The improved pharmacokinetics associated with antibody fragments in tumour penetration are often modulated by the tendency of such fragments

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to have very short circulation times *in vivo*.[6] However, enhancement of the pharmaceutical properties of antibody fragments by "PEGylation", or attachment of poly(ethylene glycol) (PEG) to the fragment, appears to confer a prolonged circulating half-life on the antibody fragments.[9–13] Protein PEGylation modulates many properties of biomedical significance, including reduced toxicity, reduced immunogenicity and antigenicity; slowing rates of clearance and proteolysis, and enhancing solubility and stability (for reviews, see Refs. 14,15).

The molecular basis of the beneficial effects of PEGylation of therapeutic proteins can be attributed to the unique physicochemical properties of PEG itself (for reviews, see Refs. 16,17). One of the most distinct features of PEG is its propensity to occupy a large volume in an aqueous environment through time-averaged water association and presents a volume 5–10 times larger than a soluble protein of comparable molecular weight,[11,14,16,18,19–21] and this can confer on a protein conjugated to it advantageous features.

Numerous functionalised PEG molecules are now available commercially.[14,18,22] An appropriate choice of the size and structure of the PEG moieties and the conjugation chemistry has been found crucial to balance the desired prolonged half-life *in vivo*, while maintaining an acceptable level of relevant biological activity or activities (including not only antigen binding and antibody effector function, but also the ability to localise to certain tissues such as tumours).[10] Hence, the molecular weight of the PEG moiety, its structure, the number and location of the PEG moieties attached to the protein, as well as the chemical method of attachment, all determine the physicochemical and pharmacological properties of the resulting protein conjugate.[10,23]

Currently there are two different kinds of PEG structure—linear or branched—which can be attached to proteins. A branched structure with a single attachment site could be more favourable than the linear structure since a high molecular weight PEG moiety can apparently be obtained without increasing the number of attachment sites.[24,25] Moreover, branched chain PEGylated proteins have been found to be more stable against enzyme proteolysis than linear moieties.[23,25] Mono-site attachment of a limited number of longer chain PEG molecules rather than multisite attachment of a greater number of smaller PEG molecules has been found most beneficial for retaining a higher level of biological activity.[9,10]

Generation of PEGylated protein requires site-specific conjugation strategies to minimise the amounts of unmated isomers produced.[19,23,26] In the case of Fab' (Fig. 1) it is possible to engineer a cysteine residue in the 'residual' hinge region of the molecule (or more than one if required): to facilitate specific PEGylation through reaction between the thiol group of the cysteine and the maleimide group present in a PEG-maleimide construct to form a stable thioether linkage.[9,10,24] This reaction can generate site-specific PEGylation of the Fab' (Fig. 2) resulting in yielding a homogeneous PEGylated product preserving the full binding activities of the antibody fragment.[9]

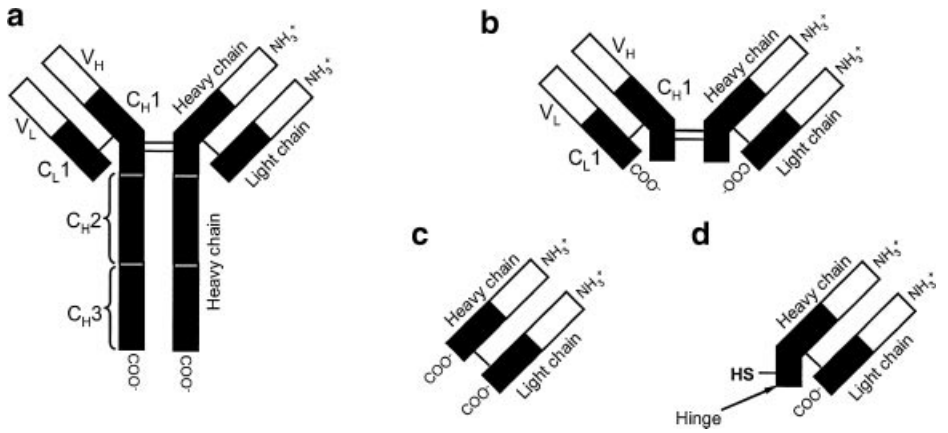

Figure 1. Schematic representation of an intact antibody molecule and some antibody fragments. (a) IgG; (b) $(Fab')_2$; (c) Fab: no hinge region, the heavy chain and light chain are linked through one disulfide bond; (d) Fab': has a short hinge region with a free cysteine.