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Research paper

Monospecific bivalent scFv-SH: Effects of linker length and location of an engineered cysteine on production, antigen binding activity and free SH accessibility

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Abstract

Development of tumor targeting pharmaceuticals on a modular platform is an attractive paradigm. Design choices for bispecific (anti-tumor and anti-chelate) pretargeting molecules are increased by the use of scFvs. Because a scFv is monovalent and small in size, its functional affinity and in vivo residence time can be improved through multimerization. ScFv multimers can be covalent or non-covalent. In vivo studies indicate that covalent scFv multimers are preferable. Attachment of scFv modules to scaffolds offers a wide range of possibilities for size and valency. A free thiol introduced at the C terminal end of a scFv (scFv-SH) allows for site-specific covalent attachment to a PEG scaffold without interfering with its antigen (Ag) binding. Although in theory, multimerization of 3 or 4 scFvs can be achieved by direct conjugation, as scFv-SH, to a tri or tetrafunctionalized PEG, it is not a practical option since homogeneous tri and tetrafunctionalized PEG are not readily available. However, the generation of $(scFv)_{3-4}$-PEG molecules through attachment of combinations of di-scFv-SH (tandemly expressed scFvs) and scFv-SH or 2 di-scFv-SH to a bifunctional PEG is a sound approach that also allows for better control of the scFv-PEG conjugate molecular composition.

Optimization of the molecular format of the di-scFv-SH module for production as soluble proteins in *E. coli*, Ag binding and conjugation is reported in this study. ScFvs in the VH-VL format were used for the di-scFv constructs since Fv domain inversion to VL-VH, while not yielding more protein, also abolished Ag binding. The effects on production yield, Ag binding and conjugation potential of the scFv joining linker length and the presence and location of an engineered cysteine were assessed in vitro. Our data indicate that for di-scFv-SH, an increase of the scFv joining linker length results in higher production and better Ag binding; a 20 aa long linker $(G_4S)_4$ was the longest linker tested. For the engineered cysteine, three locations were tested; within the scFv joining linker, at the C terminus upstream of the E Tag and as the carboxy terminal aa. The accessibility of the free SH assessed by conjugation of di-scFv-SH to HRP-Mal demonstrated that di-scFv-HRP conjugates are formed with comparable efficiencies when the cysteine is located at the scFv carboxy end.

Abbreviations: aa, amino acid; Ag, antigen; BCIP/NBT, 5-bromo 4-chloro 3-indolyl phosphate/*p*-Nitrotetrazolium Blue; di-scFv, tandemly expressed scFvs; HRP, horse radish peroxidase; IHC, immunohistochemistry; ITPG, isopropyl-b-D-thiogalactopyranoside; MAb, monoclonal antibody; MUC1, mucin 1; MW, molecular weight; OD, optical density; PBS, phosphate buffer saline; scFv, single chain Fv antibody fragment; RIT, radioimmunotherapy; TCEP, tri(2-carboxyethyl)phosphine hydrochloride; VNTR, variable number of tandem repeats.

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This empirical work provides a framework for the development of bispecific scFv multimers via site-specific attachment of scFv-SH and di-scFv-SH modules to a scaffold.

Keywords: di-scFv; Linker length; Free thiol; Site-specific conjugation

1. Introduction

Development of tumor targeting pharmaceuticals on a modular platform is an attractive paradigm to enhance the therapeutic index of pretargeted systemic radioimmunotherapy. Conformation and molecular weight (MW) of the tumor targeting molecules are critical factors that influence their pharmacokinetics; high diffusion through tumor vasculature and slow blood clearance promote accumulation at tumor sites. For a prolonged circulation time, the MW of a tumor targeting molecule should be above the maximal MW cut off for glomerular filtration estimated to be at about 70 KD (Knauf et al., 1988).

Pharmacokinetic studies have shown that a bivalent 150 KD antibody (Ab) displays low blood clearance and slow and incomplete tumor ingress and penetration (Jain, 1990). On the other hand, a monovalent 25 KD single chain Fv fragment (scFv) diffuses readily through the vasculature but is cleared too rapidly from the blood to achieve optimal tumor targeting (Yokota et al., 1992; Adams, 1998). While there are no rules for the conformation of tumor targeting molecules, their optimal combinations of MW and conformation for maximal activity have to be determined empirically with considerations of known pharmacokinetics.

Multimerization is a way to increase valency and MW of a scFv without binding specificity alterations, as well as to expand molecule conformation possibilities. ScFv multimers can be generated in several ways: by non-covalent or covalent associations, a combination of both, and by site-specific conjugation to a scaffold.

In a typical scFv, the Fv domains are stabilized through a 15 aa long, flexible and hydrophilic linker composed of 3 repeats of a G_4S unit (Huston et al., 1988). Non-covalent scFv multimerization is achieved by reducing the length of Fv joining linker to below 12 aa whereby Ag binding sites are formed by scFv intermolecular associations (Todorovska et al., 2001). The most frequent of such multimers is the dimer or diabody, that can either be mono or bispecific (Holliger et al., 1993). As a consequence of VH/VL interface stability, concentration, ionic strength, pH and expression method can influence the proportions of diabodies and higher multimers observed for a typical scFv (Desplancq et al., 1994; Arndt et al., 1998). In miniantibodies, non-covalent scFv oligomerization occurs through self-associating peptides (Pluckthun and Pack, 1997) or the use of a heterodimerization domain (Arndt et al., 2001). In "streptabodies", tetravalency is achieved by oligomerization of scFv-biotin on streptavidin (Cloutier et al., 2000). Alternatively, scFv tetramers have been obtained by expression of scFv-strepavidin fusion proteins (Kipriyanov et al., 1995; Schultz et al., 2000). Although the presence of streptavidin raises immunogenicity concerns, scFv-SA are promising pretargeting agents for RIT (Schultz et al., 2000; Cheung et al., 2004).

However, pharmacokinetic studies have shown that overall, due to their superior intrinsic stability, covalent complexes are preferable (FitzGerald et al., 1997; Cochlovius et al., 2000; Olafsen et al., 2004). Covalent bivalent, mono or bispecific scFvs are obtained either by expression of scFv genes organized in tandem (Kipriyanov et al., 1999; Kurucz et al., 1995) or by post-translational association between scFvs that carry C terminal dimerization tags. Such tags can be self-associating peptides with an engineered cysteine (Pluckthun and Pack, 1997) or merely a cysteine that provides a free thiol for disulfide bond formation (Adams et al., 1993; Wang et al., 1997; Albrecht et al., 2004; Olafsen et al., 2004) or chemical cross-linking (Cumber et al., 1992; Albrecht et al., 2004). The expression of tandem scFv genes with short linker sequences at both, domain and scFv, junctions leads to the dimerization of single chain diabodies (scDb) resulting in the formation of non-covalent, tetravalent scFvs also called tandem diabodies or tandab (Kipriyanov et al., 1999; Le Gall et al., 2004) or linear dimeric scDb (LD-scDb) (Volkel et al., 2001). Other covalent mono or bispecific scFv multimers have been assembled through the use of multimerization motifs with engineered cysteines (Pluckthun and Pack, 1997), IgG constant domains (Lu et al., 2002, 2003; Xie et al., 2003) or a combination of scFv genes expressed in tandem and multimerization motifs (Muller et al., 1998).

Site-specific conjugation of scFvs onto a scaffold, e.g. PEG, offers an alternative route for the building of multivalent covalent complexes. The therapeutic