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Site-specific photocoupling of *p*Bpa mutated scFv antibodies for use in affinity proteomics

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ABSTRACT

Recombinant antibody libraries can provide a source of renewable and high-performing binders tailored for use in affinity proteomics. In this context, the process of generating site-specific 1:1 tagging/functionalization and/or orientated surface immobilization of antibodies has, however, proved to be challenging. Hence, novel ways of generating such engineered antibodies for use in affinity proteomics could have a major impact on array performance. In this study, we have further tailored the design of human recombinant scFv antibodies for site-specific photocoupling through the use of an unnatural amino acid (UAA) and the Dock'n'Flash technology. In more detail, we have generated the 2nd generation of scFvs carrying the photoreactive UAA *p*-benzoyl-L-phenylalanine (*p*Bpa). Based on key properties, such as expression levels, activity, and affinity, a preferred choice of site for *p*Bpa, located in the beginning of the C-terminal affinity-tag, was for the first time pin-pointed. Further, the results showed that pBpa mutated antibody could be site-specifically photocoupled to free and surface immobilized β-cyclodextrin (an affinity ligand to *p*Bpa). This paves the way for use of scFv antibodies, engineered for site-specific photochemical-based tagging, functionalization, and orientated surface immobilization, in affinity proteomics.

1. Introduction

Recombinant antibody libraries can provide a diverse, customizable, and renewable source of high-performing binders tailored for use in affinity-based applications [1–5]. However, the quest of designing recombinant antibodies engineered for a specific use with respect to properties, such as affinity, stability, and on-chip functionality is challenging [2]. In the context of affinity proteomics, novel ways of providing efficient means for site-specific 1:1 tagging/functionalization and orientated surface immobilization of antibodies will be essential for improved performance [6–8].

Current methods at hand for antibody modifications range from basic approaches, such as covalent modification via exposed lysine residues, to more advanced techniques, such as the introduction of affinity tags and fusion proteins. However, all of these approaches suffer from various significant drawbacks. In the former case, the conjugation cannot be performed in a controlled and site-specific manner. This results in heterogeneously functionalized antibodies, and impaired binding activity if the modification occurs in or close to the antigen-binding-site, as well as only a fraction of the antibodies being immobilized in an orientated fashion (i.e. with their antigen-binding site facing upwards) [6]. In the latter case, the moderate binding strength provide by most affinity tag systems (e.g. His_6-tag—Ni^{2+}-NTA), the lack of matching, high-performing surfaces (e.g. Ni^{2+}-NTA) [6], and bulky fusion proteins represents key issues impairing downstream performances in affinity proteomics [2,6].

A new dimension in protein engineering was introduced, when Schultz and co-workers generated novel tRNA/aminoacyl tRNA synthetase pairs, enabling unnatural amino acids (UAAs) with new and diverse physical and biological properties to be introduced [9,10]. Noteworthy, the introduction of UAAs is compatible with downstream site-specific 1:1 conjugation of mutated antibodies (proteins) [9–11], as well as orientated surface immobilization [12]. A variety of highly selective coupling chemistries, including both affinity binding and/or

Abbreviations: β-CD, β-cyclodextrin; CDR, complementarity determining region; FW, framework; *p*Bpa, *p*-benzoyl-L-phenylalanine; scFv, single-chain Fragment variable antibody; UUA, unnatural amino acid

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Table 1
Forward and reverse primers used for site-directed mutagenesis.

Mutation	Primer sequence (5′-3′)	
Loop 1	Fwd.	GCCATCAGTGGGCTC**TAG**TCCGAGGATGAGGCT
	Rev.	AGCCTCATCCTCGGA**CTA**GAGCCCACTGATGGC
Loop 2	Fwd.	TCTGGGACCCCCGGG**TAG**AGGGTCACCATCTCT
	Rev.	AGAGATGGTGACCCT**CTA**CCCGGGGGTCCCAGA
Loop 3	Fwd.	ACGCTGTATCTGCAAATG**TAG**AGCCTGAGAGCCGAGGAC
	Rev.	GTCCTCGGCTCTCAGGCT**CTA**CATTTGCAGATACAGCGT
Loop 4	Fwd.	TCTGGGGGAGGC**TAG**GTACAGCCTGGG
	Rev.	CCCAGGCTGTAC**CTA**GCCTCCCCCAGA
Loop 5	Fwd.	CCACCCTCAGCGTCTGGG**TAGAGC**GGGCAGAGGGTCACC
	Rev.	GGTGACCCTCTGCCC**GCTCTA**CCCAGACGCTGAGGGTGG
Loop 6	Fwd.	CTGGGGGAGGCTTGGTA**TAG**CCTGGGG
	Rev.	CCCCAGG**CTA**TACCAAGCCTCCCCCAG
Tag 1	Fwd.	ACGGTCCTAGGTGAA**TAG**AAACTCATCTCAGAA
	Rev.	TTCTGAGATGAGTTT**CTA**TTCACCTAGGACCGT
Tag 2	Fwd.	GCCCATCATCATCATCATCAT**TAGAGC**TGATCACCGCGGTGGCCA
	Rev.	TGGCCACCGCGGTGATCA**GCTCTAATG**ATGATGATGATGATGGGC
Tag 3	Fwd.	GCCCATCATCATCATCATCAT**TAGAAG**TGATCACCGCGGTGGCCA
	Rev.	TGGCCACCGCGGTGATCA**CTTCTA**ATGATGATGATGATGATGGGC
Tag 4	Fwd.	CATCATCAT**TAG**AAG**GCG**TGATCACCGCGGTGGCCA
	Rev.	TGGCCACCGCGGTGATCA**CGC**CTT**CTA**ATGATGATG
Tag 5	Fwd.	CATCATCAT**TAG**AAG**GCGGCG**TGATCACCGCGGTGGCCA
	Rev.	TGGCCACCGCGGTGATCA**CGCCGC**CTT**CTA**ATGATGATG
Tag 6	Fwd.	ACCAAGCTGACGGTCCTA**TAG**GGTGAACAAAAACTCA
	Rev.	TGAGTTTTTGTTCACC**CTA**TAGGACCGTCAGCTTGGT
Tag 7	Fwd.	AAGCTGACGGTCCTAGGT**TAGAGCGGT**GAACAAAAACTCATCTCAG
	Rev.	CTGAGATGAGTTTTTGTTC**ACCGCTCTA**ACCTAGGACCGTCAGCTT

covalent binding (e.g. based on click chemistry or photochemistry) can be applied, for review see [8–14]. The use of UAA in antibodies is still, however, in an early phase and mainly explored for designing and generating site-specific antibody drug conjugates [15–17].

In this context, the use of the UAA *p*-benzoyl-L-phenylalanine (*p*Bpa) is of particular interest as it acts as a specific covalent photo cross-linker [9]. Furthermore, it serves as a guest molecule (i.e. specific binder) for the cyclic oligosaccharide β-cyclodextrin (β-CD). These unique properties are exploited with the Dock'n'Flash technology, in which *p*Bpa specifically docks into the hydrophobic cavity of β-CD, whereby an inclusion complex is formed [18]. Subsequent non-denaturing UVA-irradiation of the complex results in formation of a covalent bond and thereby one-step specific coupling without the need of having to add of any other reagents. In a recent pilot study [12], we have for the first time introduced *p*Bpa into a human recombinant single-chain Fragment variable (scFv) antibody, selected from a phage display library, through site-directed mutagenesis. The data showed that the *p*Bpa mutated scFv (Q17*p*Bpa in VL) could be expressed with retained structural and functional properties [12]. We also showed that the *p*Bpa mutated scFv could be coupled to β-CD, both in solution and onto a β-CD functionalized array slide, using the Dock'n'Flash technology.

In this study, we have expanded and further explored the design and production of *p*Bpa mutated scFv antibodies for photochemistry-based conjugation using the Dock'n'Flash methodology. In total, 4 scFv antibodies were used as model clones and 13 different positions for site-directed mutagenesis were investigated. The *p*Bpa mutated scFv were compared to their matching wild-type in terms of production levels, array activity, and apparent binding affinity as well as coupling to β-CD in solution and to a newly designed β-CD functionalized array surface. The results outlined a preferred site for mutation and confirmed that *p*Bpa mutated scFv antibodies could be site-specifically functionalized with β-CD in solution and immobilized to a β-CD functionalized surface. Hence, this paves the way for tailoring UAA modified scFv antibodies for 1:1 site-specific covalent functionalization and orientated immobilization for use in affinity proteomics, such as antibody microarrays.

2. Material and methods

2.1. Antibodies

Four human recombinant wild-type single-chain Fragment variable (scFv) antibody clones against the human complement proteins C1q (a-C1q-WT) and C3 (a-C3-WT), vascular endothelial growth factor (a-VEGF-WT), and cholera toxin subunit B (a-CT-WT) were selected from an in-house designed phage display library [19]. The scFv antibodies are based on a single, constant scaffold (V_H3-23–V_λ1-47), representing a scFv antibody design previously proven to perform well in microarray-based applications [6,7]. The scFv antibodies also carry a C-terminal affinity tag in the format of a His_6-tag, preceded by either a MYC-tag (a-C1q, a-C3, and a-VEGF) or a FLAG-tag (a-CT).

2.2. Structural modeling and in silico mutagenesis

A previously created homology model of a-C1q-WT [12] was visualized using the PyMOL Molecular Graphics System version 1.3 (Schrödinger, LLC) to identify suitable *p*Bpa mutation sites. In silico mutagenesis was performed in PyMOL using a structural file for *p*Bpa (ID: PBF) from a non-natural amino-acid sidechain database (http://www.swisssidechain.ch). The mutation sites were chosen according to the following three criteria: 1) Opposite side of the complementarity determining regions (CDR) in order to minimize any interference with antigen binding. 2) Within an exposed loop structure of the scFv framework (FW) or in the C-terminal affinity tag in order to optimize the steric accessibility to *p*Bpa and to minimize the risk of disturbing the structure of the antibody. 3) The first base after the TAG codon should be an adenine (preferred) or guanine [20], to minimize the risk of premature termination of the recombinant protein. The mutation site Q17*p*Bpa was previously created and tested in a-C1q [12]. Q17*p*Bpa is named L2 and was here also tested in all the model scFv clones (see Section 2.1) in order to evaluate the general applicability of this mutation site. In order to explore other possible mutation sites, 12 additional mutation sites were selected and evaluated in a-C1q and a-VEGF. Five of them were located in loop structures and denoted L1, and L3 to L6, and seven in the affinity tag and denoted T1 to T7. The amino