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Molecular design of recombinant scFv antibodies for site-specific photocoupling to β-cyclodextrin in solution and onto solid support

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ABSTRACT

The ability to design and tailor-make antibodies to meet the biophysical demands required by the vast range of current and future antibody-based applications within biotechnology and biomedicine will be essential. In this proof-of-concept study, we have for the first time tailored 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 successfully explored the possibility to expand the genetic code of *E. coli* and introduced the photoreactive UAA *p*-benzoyl-L-phenylalanine (*p*Bpa), and showed that the mutated scFv antibody could be expressed in *E. coli* with retained structural and functional properties, as well as binding affinity. The *p*Bpa group was then used for affinity capture of the mutated antibody by β-cyclodextrin (β-CD), which provided the hydrogen atoms to be abstracted in the subsequent photocoupling process upon irradiation at 365 nm. The results showed that the *p*Bpa mutated antibody could be site-specifically photocoupled to free and surface (array) immobilized β-CD. Taken together, this paves the way for novel means of tailoring recombinant scFv antibodies for site-specific photochemical-based tagging, functionalization and immobilization in numerous applications.

1. Introduction

The humoral immune response is mediated by antibodies, acting as highly specific binders against invading pathogens. This unique binding specificity has been exploited in a vast range of antibody-based applications in biotechnology and biomedicine. The ability to engineer antibodies to meet the biophysical demands required by these applications, such as specificity, affinity, and stability [1,2] is critical. Adopting recombinant antibody libraries, representing large, renewable sources of high-performing binders [3,4], will open up new avenues for generating, designing, and tailoring binders with such desired properties [5–7].

Engineering antibodies with improved properties, such as stability, have traditionally been accomplished by mutating single or combinations of key amino acid residues [8–10], limiting the building blocks to the standard 20 amino acids. By generating novel transfer RNA (tRNA)/aminoacyl-tRNA synthetase pairs, Schultz et al. have successfully expanded the genetic code, and made it possible to genetically encode for more than 70 unnatural amino acids (UAAs) with novel and diverse physical, chemical, or biological properties in *Escherichia coli* (*E. coli*), yeast, and mammalian cells [11,12]. These novel building blocks include fluorescent, glycosylated, metal-ion-binding, and redox-active amino acids, as well as amino acids with unique chemical and photochemical reactivity [13,14], for review see [15], providing a powerful method for generating proteins with novel and/or enhanced properties. In the case of antibodies, UAA has so far been introduced to full length IgG and/or fragment antigen-binding (Fab) thereof, to generate e.g. dimeric antibodies [16], multimeric antibodies [17], antibody–drug conjugates [18], and antibody–DNA conjugates [19].

Introducing photoreactive groups in antibodies (proteins) could provide new means for tagging, functionalization, and/or surface immobilization [20–23]. To date, this has mainly been accomplished using photonic activation of accessible disulfide bridges, as illustrated for Fab antibody fragments [20,21,23]. An alternative route could be to use UAAs instead, such as the photo cross-linker *p*-benzoyl-L-phenylalanine

Abbreviations: β-CD, β-cyclodextrin; *p*Bpa, *p*-benzoyl-L-phenylalanine; Fab, fragment antigen-binding; FW-1, framework-1; MW, molecular weight; scFv, single-chain Fv variable; T_m, melting temperature; tRNA, transfer RNA; UAA, unnatural amino acids; RT, room temperature; VEGF, endothelial growth factor; VH, variable domain oft he heavy chain; VL, variable domain of the light chain

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(*p*Bpa), which bears the photoreactive benzophenone moiety [11,13,14]. The ketyl group of *p*Bpa typically reacts with C – H bonds upon excitation of near-UV light (350–360 nm) resulting in the formation of an intramolecular C – C covalent bond between reacted *p*Bpa and target molecule. Based on this, the dock'n'flash technology was recently developed providing the means to specifically and covalently photocouple *p*Bpa mutated proteins with site-specific control based on a bioaffinity mechanism [24]. In more detail, the authors successfully exploited the affinity docking of the *p*Bpa group at the protein surface into a β-cyclodextrin (β-CD) host, which were then covalently coupled in a light-dependent reaction with non-denaturing UVA, using cutinase (*Fusarium Solani Pisi* cutinase) as model protein.

In this proof-of-concept study, we have adopted the dock'n'flash technology and for the first time introduced the photoreactive UAA *p*Bpa residue into a single-chain Fv variable (scFv) antibody, selected from a human recombinant antibody phage display library, engineered around a single, constant framework (V_H3-23–V_λ1-47) [3]. The results showed that the *p*Bpa mutated scFv could be expressed with retained structural and functional properties, including binding affinity, and that the mutant could be site-specifically photocoupled to β-CD in both solution and on-chip (array immobilized). Hence, this opens up novel routes for tailoring scFv antibodies for photochemical-based tagging, functionalization, and immobilization in a wide range of applications, exemplified by, but not limited to, antibody-based microarrays [5,25].

2. Material and methods

2.1. Antibodies

A human recombinant wild-type scFv antibody, denoted a-C1q-wt, directed against the human complement protein C1q, was selected from an in-house designed phage display library, as previously described [3]. The a-C1q-wt antibody was selected as a model antibody, since it has been demonstrated to display high specificity, affinity (K_d of 2×10^{-10} M), stability, and on-chip microarray functionality [26–28]. In a similar manner, an endothelial growth factor (VEGF) specific scFv antibody, denoted a-VEGF, was selected and used as negative control.

2.2. Site-directed mutagenesis

The position of the *p*Bpa (Bachem, Bubendorf, Switzerland) mutation was chosen according to the following three criteria: 1) Far from the complementarity determining regions (CDR) in order to minimize any interference with antigen binding. 2) Within an exposed loop structure in order to maximize the access 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, to minimize the risk of premature termination of the recombinant protein [29]. To this end, a *p*Bpa mutation site was defined in the variable domain of the light chain (VL), Q17, of a-C1q-wt.

Gene encoding *p*Bpa mutated a-C1q-wt, denoted a-C1q-mutant, was generated using QuikChange®Site Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) following the instructions provided by the manufacturer. A complementary forward (5′ TCT GGG ACC CCC GGG **TAG** AGG GTC ACC ATC TCT 3′) and reverse (5′AGA GAT GGT GAC CCT **CTA** CCC GGG GGT CCC AGA 3′) substitution mutation primer pair was designed, and ordered from Eurofins MWG (Edersberg, Germany). The mutated gene construct carried in the pFab5C.his vector was then transformed into competent *E. coli* XL1-Blue competent cells. Glycerol stocks were generated and stored at −80 °C until further use. Mutagenesis was verified by DNA sequencing (Eurofins MWG Operon, Ebersberg, Germany).

2.3. Co-transformation

The a-C1q-mutant-coding plasmid was co-transformed with a supplementary plasmid, pSUP*p*Bpa-6TRN plasmid encoding the *p*Bpa tRNA/aminoacyl tRNA synthetase pair, a gift from Dr. Peter Schultz, Scripps Institute, La Jolla, CA, USA, into the production host TOP10F′ *E. coli*. Successful clones were re-sequenced (EuroFins MWG Operon) and stored as glycerol stocks at −80 °C until further use.

2.4. Antibody production

The a-C1q-wt, a-VEGF (100 μg/ml ampicillin), and a-C1q-mutant (100 μg/ml ampicillin and 25 μg/ml chloramphenicol) scFv antibodies were produced in *E. coli* (100 ml cultures). In the case of a-C1q-mutant, the growth medium (2xYT) was supplemented with 1 mM *p*Bpa [29]. Due to the light sensitivity of *p*Bpa all handling of *p*Bpa-containing preparations were performed in laboratory areas without windows and lighting. The produced scFvs were purified from either supernatant or periplasmic preparations using affinity chromatography on Ni^{2+}-NTA agarose gel (Qiagen, Hilden, Germany). Bound molecules were eluted with 1 ml 250 mM imidazole, extensively dialyzed against PBS, and stored at 4 °C, until further use. The protein concentration was determined by measuring the absorbance at 280 nm using NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, USA). The degree of purity and integrity of the scFvs was evaluated using 10% SDS-PAGE (Invitrogen, Carlsbad, CA, USA) and Western-blot. In the latter case, the scFv antibodies were visualized using mouse α-MYC antibody (Zymed, San Francisco, CA, USA) and horseradish peroxidase conjugated rabbit-anti-mouse immunoglobulin (Dako Denmark A/S, Glostrup, Denmark), and developed in a KODAK X-OMAT 1000 processor (Kodak Nordic AB, Upplands Väsby, Sweden) with Amersham Hyperfilm™ ECL (GE Healthcare).

2.5. MALDI-TOF analysis

The antibody preparations were cleaned using $ZipTip_{C4}$ Pipette Tips (Millipore, Billerica, MA, USA) before adding the matrix. The tips were equilibrated by washing with 50% (*v*/*v*) acetonitrile (ACN) followed by 0.1% (*v*/*v*) TFA. Bound proteins were washed with 0.1% (*v*/*v*) TFA, and subsequently eluted with 70% (*v*/*v*) ACN 0.1% (*v*/*v*) TFA. The eluates were mixed 1:1 with matrix (10 mg/ml cinnamic acid, 40% (*v*/*v*) ACN, 0.06% (*v*/*v*) TFA) and then manually dispensed as double droplets (1 μl/drop) onto a solid support. The first droplet was allowed to dry before spotting the second. Due to problems purifying the a-C1q-mutant-CD with $ZipTip_{C4}$ tips, solutions were directly mixed with the above-mentioned matrix and spotted. Dried samples were analyzed using MALDI micro MX ™ (Waters, Milford, MA, USA) and MassLynx™ Software (Waters).

2.6. Circular dichroism

The secondary structure and thermostability of the scFv antibodies were examined using circular dichroism (CD), (J-720 Spectropolarimeter (Jasco, Eaton, MD, USA)). The scFvs were dialyzed (MWCO 12-14.000) against PBS for 1–3 days to remove sodium azide. The samples were scanned at 20 °C in the far UV, 250–200 nm. A temperature scan from 20 °C to 95 °C with a thermal ramp of 1 °C/min was also performed, and change in molar ellipticity at 218 nm was recorded, with two data points per minute. Thermal unfolding scans were analyzed assuming a two-state equilibrium between native and denatured state, and the mid-point of the transition, T_m-value (melting temperature), was estimated.