PEGYLATION AND MULTIMERIZATION OF THE ANTI-P185HER-2 SINGLE-CHAIN FV FRAGMENT 4D5: EFFECTS ON TUMOR TARGETING*

SUPPLEMENTARY METHODS

Susanne Kubetzko*, Ela Balic*, Robert Waibel& Uwe Zangemeister-Wittke†§* and Andreas Plückthun‡

From the †Department of Biochemistry, University of Zürich, CH-8057 Zürich; §Department of Oncology, University Hospital, University of Zürich, CH-8044 Zürich; †Paul Scherrer Institute, CH-5232 Villigen (PSI), Switzerland

Running title: ScFv PEGylation and Multimerization for Tumor Targeting

Address correspondence to: Andreas Plückthun, Dept. of Biochemistry, University of Zürich, CH-8057 Zürich, Switzerland; Tel. +41-44-635 5570; FAX +41-44-635 5712; E-mail: plueckthun@bioc.unizh.ch

Expression and Purification — All antibody constructs were expressed in the periplasm of the E. coli strain SB536 (1), which is devoid of the periplasmic proteases HhoA and HhoB. Freshly transformed cells were grown overnight and 1 l 2YT medium containing 50 µg/ml ampicillin was inoculated with 25 ml of this starter culture. The culture was grown in a 5 l baffled shake flask at 25°C. Expression of the antibody fragments was under the control of a lac promoter (2) and induced with 1 mM (final concentration) isopropyl-β-D-thiogalactopyranoside (IPTG, Eurogentec) at an OD

Expression was allowed to continue for 4 to 6 h until the culture reached a final OD

Cells were harvested by centrifugation at 4000 g for 15 min at 4°C and stored as a pellet at −80°C.

Purification of the antibody fragments was carried out by two subsequent affinity chromatography steps on a GradiFrac system (Pharmacia) at 4°C. The harvested E. coli cells were resuspended in 20 mM Tris (pH 7.4) buffer, containing 150 mM NaCl, 4 mM MgCl\(_2\) and DNase (1 mg/100 ml), and then passed through a French Press (SLS Instrument Inc., Urbana Illinois, USA) at 10,000 p.s.i. for cell lysis. The lysate was immediately centrifuged in a SS-34 rotor at 48,000g for 30 min at 4°C to pellet cell debris. The filtered (0.22 µm) lysate was then loaded onto a Ni-NTA Superflow column (Qiagen) and washed with running buffer (20 mM Tris, pH 7.4, 150 mM NaCl) until the absorption (280 nm) reached the baseline. After two additional washing steps, first with 1 M NaCl and then with 40 mM imidazole, the bound protein was eluted with 200 mM imidazole (pH 7.4). The eluted sample was applied to a Protein A Sepharose column (Pharmacia), equilibrated with the same running buffer as used for the Ni-NTA column. After the absorption (280 nm) of the column flow-through had reached the baseline again, the bound miniantibody was eluted with 100 mM citrate buffer, pH 3.5. The collected sample was immediately neutralized by titration with a 1 M Tris solution (200 µl/ 1 ml eluate), which was performed on each fraction upon elution. The quality of the purified protein was analyzed by UV-spectrometry and SDS-PAGE (12% gel, Coomassie staining).

PEGylation of the 4D5-miniantibodies (3) — Purified protein samples of the monomeric (scFv 4D5-Cys)
and dimeric (4D5-dhlx-Cys) miniantibodies were concentrated to approximately 0.3 to 1 mg/ml by centrifugation at 2000g and 4°C, using a 10-kDa cutoff microconcentrator (Ultrafree-MC low protein binding; Millipore, Billerica, MA). To enable site-specific PEGylation, the C-terminal cysteine residue was selectively reduced before incubation with maleimide-PEG20 (Nektar, Huntsville, AL). The reducing conditions had to be mild to prevent breakage of the internal disulfide bonds in the V_L and V_H domains. Therefore, the concentrated protein solution was incubated with 3 mM dithiothreitol (final concentration) for 30 min at 37°C. The excess reducing agent was then immediately removed by desalting over a Sephadex G-25 column (PD-10 or NAP-5; GE Healthcare, Little Chalfont, Buckinghamshire, UK). In this step, the buffer was also exchanged to the one used in the PEGylation reaction (100 mM citric acid, 100 mM NH_4OAc, 2 mM EDTA, pH 6.0, filtered, degassed and saturated with N_2). The PEGylation reaction was then carried out by addition of maleimide-PEG20 in 5- to 10-fold molar excess over freshly reduced protein, followed by incubation at 37°C for 2 h. The efficiency of PEGylation was analyzed by SDS-PAGE (12% gel, Coomassie-staining) and size exclusion chromatography on a Superdex-200 column with an ÄKTAexplorer system (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The same chromatography system was used to purify the PEGylated proteins from both the native antibody fragments and the unreacted free PEG.

References