

Engineering recombinant antibodies for polymer biofunctionalization

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ABSTRACT

The attachment of recognition elements such as antibody fragments to polymeric substrates can be used to mediate cell- or protein-specific interactions. In this work, single-chain Fv (scFv) antibody fragments were isolated against two cell types of interest and expressed in an *Escherichia coli* expression platform. The scFvs were engineered at their C-terminus to incorporate a cysteine-containing linker, for reaction with maleimide-linked polymers, or a heptasaccharide glycan for complexation with surface amine moieties. Antigen binding of the modified scFvs was unchanged and expression yields of the glyco-engineered scFvs were similar to the unmodified molecules, while cys-tagged scFv yields varied between scFv variants. Targeted immobilisation of the scFvs via either modification resulted in 3-5 fold higher binding of ligands over adsorbed molecules. The study a simple and efficient antibody engineering and modification approach for effective targeted immobilization on polymeric substrates.

Keywords: recombinant protein; antibody fragment; immobilization; biofunctionalization

INTRODUCTION

A large number of synthetic and biomimetic polymeric materials are in current use and in development for medical applications in fields such as prostheses, tissue engineering and drug delivery.^[1, 2] While some are designed to be chemically inert and aim to avoid rather than promote interactions with cells and other tissue components, in increasing numbers of applications, cell-recognition elements are desirable to promote and regulate interactions with specific target cells *in vivo*.^[3-5]

The high degree of selectivity of antibodies for their ligands lends them to application in creating such addition-of-function polymeric materials. Synthetic^[6,7] or cloned^[8,9] antibody libraries and recombinant expression platforms,^[10,11] honed throughout the past two decades, have abrogated the previous requirement for animals to raise antibodies and have made human antibodies as easy to produce for *in vivo* applications as non-human molecules.^[12] Furthermore, smaller, antibody-derived fragments that retain the binding pockets and properties of whole immunoglobulin molecules are readily (and cheaply) produced in bacterial systems.^[13] These fragments allow relatively simple engineering to modify properties such as affinity,^[14,15] or to introduce novel characteristics such as fusion to a cytotoxic or modification for attachment to payload-containing nanoparticles.^[3,16,18]

Proteins are susceptible to loss of activity upon immobilization on polymers or other surfaces, however.^[19] In addition, non-site-specific adsorption of proteins to polymers, or attachment via functional groups that are not unique, such as amino or carboxyl groups, has the potential to yield a heterogeneous mixture of attached proteins with differing orientations and activities,^[20-22] with as few as 10% of non-specifically adsorbed proteins on surfaces found to be active.^[23,24] Meanwhile, immobilization via non-covalent interactions can lead to leaching of the cell-targeting moiety and loss of the polymer functionality *in vivo*.^[25]

In order to achieve controlled attachment and orientation of a cell-targeting or cell-capturing moiety on a polymer surface, therefore, the use of a unique chemical attachment group on the protein molecule is often preferred.^[26] This group can be introduced at the genetic level by cloning or it can be added post-translationally to the recombinant protein *in vivo* or *in vitro* (Fig. 1).^[3] Such groups and their positioning in the molecule must be carefully designed to avoid disturbing the functional conformation or active site of the protein while allowing specific, preferably covalent, reaction with a target chemical moiety on the polymer.^[27,28]

We describe a platform technology to enable isolation of cell-targeting scFv antibody fragments and their engineering to enable covalent, site-specific immobilization interactions with appropriately prepared or modified supports, and examples of their attachment to polymeric surfaces.

EXPERIMENTAL

Bacterial strains and plasmids

Escherichia coli TG1 was utilised for phage display of antibody fragments and *E. coli* TOP10 and CLM37 for expression of soluble scFv fragments in unglycosylated and glycosylated forms, respectively. Domoic acid-binding 2H12 scFv^[27] and fluorescein-binding 4M5.3 scFv^[28] were cloned as described elsewhere. Cysteine-tagged variants of scFvs were constructed using an overlap PCR approach^[29] to generate cysteine-containing linkers described in^[27] and linkers containing glycosylation target sequences were added to scFvs using the same approach.^[28] Antibody fragments were expressed in the *E. coli* periplasm

using the pIG6 vector^[30] while the pACYC-*pgl* plasmid,^[31] kindly provided by Professor Markus Aebi, ETH Zurich, Switzerland was co-transformed into cells to allow glycosylation of scFvs by the encoded *Campylobacter jejuni* protein glycosylation machinery. The YamoI human scFv phage display library^[9] was provided by Montarop Yamabhai, Suranaree University of Technology, Thailand.

ScFv isolation

ScFvs specific for two cell types of interest were isolated from a naïve human scFv combinatorial library using a phage display approach.^[9] In the case of intervertebral disc (IVD) cells, library panning was carried out against immunoglobulin-like domain 1 of the IVD cell surface neural cell adhesion molecule 1 (NCAM1).^[32] This NCAM1-Ig1 domain was expressed in *E. coli* and purified as described elsewhere,^[33] followed by adsorption to Maxisorp immunotubes (Nunc) at 1 mg (500 µg/ml; round 1) or 100 µg (50 µg/ml; subsequent rounds) in 1% BSA/PBS at 4°C overnight. After three washes with PBS, immunotubes were blocked with 3% BSA in PBS for 2 h at room temperature. Following washing as before, 10¹² phage displaying human scFvs were added to tubes and allowed to bind the immobilised protein domain for 2 h at room temperature. After 20 washes with PBS containing Tween 20 (8 washes with 0.1%, 7 with 0.2%, 5 with 0.5% Tween) and 20 with PBS, bound phage particles were eluted by the addition of 1 mg/ml trypsin in PBS for 10 min to proteolyse phage particles from immobilized scFvs and 50 mM glycine-HCl (pH 2.0) for 10 min to break the scFv-antigen interaction. The eluate was neutralized using 200 mM Na₂HPO₄ (pH 7.5) and the eluted phage particles were used to infect *E. coli* cells, rescued and titred using standard procedures.^[9] Three rounds of panning and infection were carried out.

To isolate scFvs binding human endothelial cells, the YamoI library was panned as described above but against commercially available human VEGFR2 (Sino Biological, Beijing, China), using 100 µg, 50 µg and 50 µg VEGFR2 immobilised per Maxisorp tube in successive rounds of panning. ScFv isolation and bacterial reinfection was carried out as outlined for NCAM1-Ig1.

Analysis of NCAM1-Ig1 or VEGFR2 binding by the respective polyclonal phage populations was investigated by enzyme-linked immunosorbent assay (ELISA) using a horse radish peroxidase (HRP)-conjugated mouse anti-M13 antibody (GE Healthcare) to detect bacteriophage particles. Phage input/output ratios were used to determine scFv enrichment in each panning round.^[33] To identify target-binding monoclonal scFv-phage, 92 randomly selected clones were investigated by ELISA in the case of NCAM1-Ig1, and 94 screened by restriction profiling in the case of VEGFR2 to identify unique clones, prior to expression of scFvs from individual clones in soluble (unfused) format in the non-suppressor *E. coli* TOP10 strain. This was followed by ELISA detection of the soluble scFv fragments using a HRP-conjugated mouse IgG (Abcam), diluted 1:1,000 in PBS, for signal reporting.

Protein expression and analysis

Expression of recombinant proteins was initially investigated for all proteins in *E. coli* TOP10 and *E. coli* BL21 cells using an IPTG-based induction of expression^[34] in LB medium over 4-24 h at 25°C and by an autoinduction method^[35] in ZYP-5052 medium for 24-48 h at 25°C. Cell lysis and fractionation were carried out on harvested cells as described previously^[36] and proteins from cell lysates were analysed by SDS-PAGE and Coomassie

blue staining to compare yields and solubilities arising from different expression procedures. Immunoblotting and scFv detection followed standard procedures using a HRP-conjugated anti-polyhistidine antibody to detect the hexahistidine-tagged recombinant proteins.^[36] ScFv proteins were purified from soluble protein fractions by immobilised metal affinity chromatography (IMAC), exploiting the interaction of the hexahistidine tag with an immobilised nickel resin.^[34]

For glycosylated scFvs, glycans were detected using a DIG Glycan Differentiation kit (Roche Applied Science). This was carried out according to the manufacturer's instructions with the exception that nitrocellulose membranes were blocked for 1 h at room temperature using 2% gelatin in Tris-buffered saline containing 0.05% Tween (TBST), followed by incubation with digoxigenin-labelled *Maackia amurensis* agglutinin (MAA) in TBST containing 1 mM MgCl₂, 1 mM MnCl₂, 1 mM CaCl₂ (pH 7.5). Signal was developed after interaction of the alkaline phosphatase-conjugated anti-digoxigenin reporter antibody with membrane-attached MAA.

ELISA and kinetic analysis

Purified proteins were quantified using a Bradford protein assay kit. Cysteine-linked scFvs were added to the wells of maleimide-activated multiwell plates (Pierce) in 100 mM phosphate, 150 mM NaCl, 10 mM EDTA (pH 7.2) and incubated overnight at 4°C. After washing, wells were blocked for 2 h at room temperature with 10 µg/ml cysteine in the same buffer and subjected to three washes. In the case of glycosylated and unmodified scFvs, proteins were adsorbed onto multiwell polystyrene plates overnight at 4°C and wells were blocked using 2% BSA in PBS, followed by three washes with PBS containing 0.05% Tween (PBST). For competitive ELISAs with 2H12 scFv, domoic acid-HRP tracer (Mercury Science) was mixed with free domoic acid at concentrations from 1 to 50,000 ng/ml and added to wells, while for fluorescein-binding assays, FITC-HRP was mixed with free fluorescein at concentrations ranging from 0 to 100 ng/ml prior to addition to the wells. Binding was detected and the signal developed as described previously.^[27,28] OriginPro8 was used to fit the data and to calculate IC₅₀ values for the scFvs.

Immunocytochemistry

Due to their surface expression of NCAM1,^[37] primary rat astrocytes were used for initial screening of NCAM1-binding scFvs. The cells were isolated, seeded and cultured to 70-80% confluence as described previously.^[33] Following incubation with 5 µg NCAM1-binding F4 scFv or a control scFv, cell-associated scFvs were detected using murine anti-polyhistidine and FITC-conjugated goat anti-mouse antibodies in turn (Molecular Probes, USA).^[33] Cell nuclei were stained using DAPI and imaging was performed using an Olympus IX81 inverted epifluorescent microscope.

In the case of endothelial cell-binding scFv 32, an endothelial progenitor cell (EPC) line derived from human cord blood [38] was used to screen scFv binding. Cells were cultured in OPTIMEM medium supplemented with 3% foetal bovine serum, 1% penicillin/streptomycin and 1% L-glutamine, followed by incubation with 1 µg per well of VEGFR2-binding scFvs. Cells were blocked using 10% goat serum and detection of cell-bound scFvs was carried out using a DyLight 488-conjugated 6x-His Epitope Tag antibody (Pierce Antibodies), diluted 1:200 in PBS, prior to imaging.

FACS

Primary rat astrocytes (Passage 5) were grown in 75 cm³ tissue culture flasks up to 80% confluence. Cells were harvested by trypsinization and resuspended in DMEM-F12 media containing 10% fetal bovine serum and 1% penicillin/streptomycin. The cells were again collected by centrifugation and washed with PBS, followed by resuspension of 1×10⁶ cells in 200 µl PBS. After incubation with 10 µg/ml anti-NCAM1 F4 scFv overnight at 4°C, the cell suspension was washed using 3 ml PBS and centrifuged for 5 min at 700 g at 4°C. The cells were resuspended in 200 µl of PBS and DyLight488-conjugated anti-6His antibody (5 µg/ml in PBS) was added. The cells were again incubated for 1 h at room temperature, washed using 3 ml PBS and centrifuged as before. After a final wash to ensure complete removal of unbound antibody, the cells were resuspended in 500 µl PBS and incubated on ice for 10 min prior to the addition of 500 µl of hypotonic PI solution and FACS analysis using a BD Canto A while gating the cell population in unstained cells. Positive cells were observed in the FL1 channel and their percentage calculated using the instrument software.

RESULTS

Isolation of cell-binding antibody fragments

Screening of the YamoI human scFv library against the recombinant Ig1 domain of human NCAM1 led to a clear increase in antigen binding by eluted scFv-phage particles over rounds 2 and 3 (Fig. 2a), indicating potential enrichment for binders to the immobilised protein domain. Upon individual screening of 92 randomly selected clones from screening round 3, one clone – F4 – was identified as the highest binder in three repeated ELISAs. DNA sequencing revealed an amber stop codon in framework 2 of V_H, which was mutated to alanine by site-directed mutagenesis to allow soluble, non-phage displayed expression of the scFv in the non-suppressor *E. coli* strain TOP10 for binding analysis.

After confirmation of binding of the F4 scFv to the purified recombinant NCAM1-Ig1 domain in ELISA, investigation of its binding to the full-length, cell-bound NCAM1-protein was carried out using NCAM1-expressing rat astrocytes.^[37] The scFv was found to bind NCAM1-expressing rat astrocytes (Fig. 2b) and presented a similar cell labelling pattern to a commercial anti-NCAM1 murine monoclonal antibody (not shown). Meanwhile, the specific binding of F4 scFv to astrocytes was further confirmed by FACS, in which a positive shift in the FL1 channel was observed and approximately 50% of the cell population exhibited scFv binding (Fig. 2c), while no binding was detected in the isotype control.

A similar phage display approach was used to isolate scFvs with binding to endothelial progenitor cells. Antibody library screening was carried out on purified, immobilized VEGFR2 protein, with a marked increase in antigen binding by the polyclonal phage population again noted after three panning rounds (not shown). As the bacteriophage clones were isolated based on their binding to non-cell associated antigen, clones which exhibited VEGFR2 binding *in vitro* were further screened by immunocytochemistry using EPCs to confirm their ability to recognize the full-length, cell surface-displayed receptor. Of the evaluated scFvs, clone 32 was selected for further investigation due to its consistently strong binding to the human EPCs (Figure 2d).

Functionalization and expression of scFvs

To produce and harvest antibody fragments for polymer attachment, recombinant expression of the proteins in reasonable yields of correctly folded, active form is necessary. Three model scFvs (2H12, binding domoic acid; 32, binding VEGFR2; 4M5.3, binding fluorescein) were successfully cloned with C-terminal glycosylation or cysteine-containing recombinant tags to facilitate their interaction with polymers and solid supports, in order to investigate the effects of the tags on their expression and/or target binding (Fig. 3a). All constructs also contained a protease (TEV)-removable hexahistidine tag ("6H") for ease of detection and purification of expressed proteins from bacterial lysates (Fig. 3a).

While glycosylation of the engineered C-terminal amino acid motif required maintenance of a co-transformed plasmid and production of additional, heterologous protein glycosylation enzymes in the host cells (Fig. 3b), purified protein yields in the range of 1-2 mg per litre of bacterial culture were repeatedly achieved for the glycosylated fluorescein-binding 4M5.3 and domoic acid-binding 2H12 scFvs, similar to their unmodified counterparts. Though the efficiency of glycan attachment to the translated scFvs varied with physiological parameters,^[28] optimization of expression, particularly by increasing antibiotic selection for the scFv-expressing plasmid, resulted in all detectable scFvs being produced in the glycosylated format (Fig. 2c). The increase in molecular weight of the scFv is due to the addition of the glycosylation motif, which was confirmed by lectin blotting (Fig. 3c). In similar investigations with VEGFR2-binding scFv 32, obtained yields of purified proteins were in the range of 100-300 µg of scFv per litre of bacterial culture and were again unaffected by glycosylation of the scFv.

In the case of cysteine linker-containing scFvs 4M5.3 and 2H12, different expression protocols were identified for optimal expression of the different proteins. 4M5.3 scFvs with and without C-terminal cys-linkers both exhibited increased yields using autoinduction rather than IPTG-based expression procedure but yields of the cys-tagged scFv were considerably increased upon co-overexpression in the host bacterial cells of *E. coli* Hsp60 family chaperones GroES-GroEL and Hsp70 chaperones DnaK-DnaJ-GrpE to assist with protein folding and reduce protein aggregation. This resulted in similar yields of the cys-tagged and unmodified 4M5.3 scFvs (300-400 µg per l bacterial culture). This was unlike results with 2H12 scFv, however, where purified yields were 10-fold lower in the case of the cysteine-tagged scFv, at 337 µg/l compared with 3.15 mg per l bacterial culture of the unmodified scFv.^[27] In both cases, the cysteine-tagged scFvs exhibited a tendency to dimerize through the free cysteine (Fig. 3d).

Antigen binding by functionalized scFvs

The effects of the added functional moieties on ligand binding by the scFvs was investigated by competitive immunoassay with fluorescein-binding 4M5.3 and domoic acid-binding 2H12 scFvs, and by immunocytochemistry with VEGFR2-binding scFv 32. In the case of 4M5.3 scFv, no significant effect was observed on fluorescein binding due to the presence of either the attached glycan chain or the linked cysteine-containing peptide, as evaluated by competitive ELISA. The unglycosylated scFv had an IC₅₀ for fluorescein of 0.98 ng/ml and the glycosylated protein an IC₅₀ of 1.12 ng/ml (Fig. 4a), reflecting previously reported results,^[28] while the C-terminal cysteine-tag also had no detectable effect on binding, with an IC₅₀ of 0.96 ng/ml fluorescein calculated for this modified scFv.

For 2H12 scFv (Fig. 4b), the IC₅₀s for domoic acid binding of antibodies adsorbed on MaxiSorp plates and oriented, covalently immobilized cysteine-tagged antibodies were 540 and 1482 ng/ml, respectively, while the cysteine-tagged scFv had an IC₅₀ for domoic acid of

297 ng/ml when covalently immobilized on maleimide activated plates, compared with a previously reported 245 ng/ml for the scFv in solution.^[27] Meanwhile, the same scFv with a C-terminal glycosylation chain, also adsorbed on Maxisorp plates, exhibited an IC₅₀ of 680 ng/ml for domoic acid.

In the case of the VEGFR2-binding scFv 32, a semi-quantitative immunocytochemistry-based comparison of binding of glycosylated and unglycosylated scFvs to EPCs detected no reduction in cell binding signal or change in binding pattern as a result of glycosylation (Figure 4c). The apparent increase in signal intensity with the glycosylated protein was most likely due to a difference in concentration of the two proteins, caused by an observed degradation of the unmodified scFv during extended storage prior to analysis.

Immobilization on polymers and analysis

Immobilization on polymeric substrates via added functional groups was carried out with domoic acid-binding 2H12 scFv and fluorescein-binding 4M5.3 scFv. In the case of the former, 2H12 scFvs with a C-terminal cysteine-containing linker exhibited an IC₅₀ of 256 ng/ml domoic acid, improving more than 5-fold over the equivalent physisorbed scFv and 2-fold over the adsorbed, unmodified scFv (Fig. 5a). Immobilization of the unmodified scFv was significantly lower than that of the cysteine-linked protein on the maleimide plates. Meanwhile in the case of fluorescein-binding 4M5.3 scFv, antibody fragments immobilized on aminated EDA-agarose via a C-terminal glycan tag captured one fluorescein ligand per 3-5 scFvs. Adsorbed 4M5.3 scFvs, however, bound an antigen only once in every 11-19 adsorbed scFvs, or a 3-4-fold reduced efficiency of antigen binding compared with the covalently immobilized molecules.

DISCUSSION

Polymeric materials exhibit enormous potential application in a broad spectrum of biotechnological and biomedical fields. In many applications such as drug delivery, cell capture and surface coating, the ability to regulate and effect interactions with specific cell types of biomolecules is advantageous. While cell adhesive proteins such as fibronectin or collagen have been used to mediate cell binding,^[39] their usefulness is limited by their lack of specificity for particular cell types and, in some cases, the difficulty in their recombinant expression due to their complexity. In this work we describe the isolation and engineering of scFv antibody fragments to generate molecules compatible with covalent attachment to polymers for such addition-of-function applications.

While antibodies exhibit specific binding and immunocompatibility for *in vivo* applications, the traditional requirement for immunization to achieve high affinity binders, coupled with technical and ethical difficulties in raising human antibodies, has limited their application in many fields. The development of technologies such as phage^[40] and ribosome^[41] display which facilitate the isolation of human-derived antibodies of desired binding specificities using *in vitro* technologies, and the emergence of platforms to express and engineer smaller-sized, recombinant, antibody-derived fragments which retain the binding specificity of the parent molecules, has revolutionized our capability in the field of antibody engineering and, therefore, targeting and diagnostic applications.

An outstanding limitation of the use of antibody fragments in applications such as *in vitro* diagnostics and *in vivo* targeted therapy has been the tendency of proteins, including antibodies, to undergo unfolding processes and lose function on solid or polymeric

supports.^[19] Non-site-specific immobilisation approaches also typically yield heterogeneous mixtures of orientations, folded states of proteins that further limit their activity in the bound state.^[22] Therefore, it becomes necessary to design a targeted immobilisation approach which is easy to carry out, does not incur additional production costs, and, critically, does not affect the conformation or function of the immobilized protein. Methods should not modify the conformation or activity of the engineered protein, as well as not introducing new bottlenecks to limit their folding or expression in the recombinant expression host. Modification of scFvs with a uniquely reactive, surface-exposed functionality can be carried out at N- or C-terminal domains, or occasionally in the inter-domain linker,^[42] such that the overall conformation of the protein is unaffected. C-terminal engineering is frequently preferred due to its typical lack of impact on expression and on subcellular trafficking processes associated with N-terminal signal sequences in many recombinant expression hosts.

ScFvs were modified at the C-terminus in this work via the addition of either a cysteine-containing linker, added during polypeptide translation, or a glycosylation moiety, with post-translation addition of a heptameric sugar chain to the motif in the host bacterial cells. While the latter was expected to reduce the scFv yield or folding efficiency in *E. coli* due to the increased metabolic burden of plasmid maintenance and expression of the heterologous protein glycosylation machinery, all glycosylated scFvs were expressed and purified at levels comparable with their unglycosylated homologues, and with no additional costs associated with the fermentation. Cysteine linker-containing scFvs, meanwhile, exhibited a greater variability in expression, ranging from yields that were also comparable with their unmodified counterparts to an approximate ten-fold reduction in soluble, functional yields. This reduction is unsurprising as proteins containing multiple cysteines present particular expression problems for *E. coli*, and particularly with proteins containing non-disulfide-linked cysteines,^[43] as in the case of the engineered scFvs. A further problem encountered with cysteine-tagged scFvs was their tendency to dimerize upon purification. As this would impede their complexation with thiol-containing surfaces, purified proteins had to be stored under reducing conditions to avoid dimerization, followed by exchange for oxidizing conditions when incubated with supports for immobilization.

Immobilized scFvs exhibited significantly improved ligand binding over their adsorbed counterparts. Domoic acid binding improved more than 5-fold upon targeted immobilization via a cys-linker, while similarly, the specific binding activity of fluorescein binding of 4M5.3 scFvs increased 3-5-fold in directed, covalently immobilized scFvs compared with physisorbed molecules. These improvements in binding in the targeted immobilized scFvs may be due to improved accessibility of bonding pockets, reduced steric hindrance between molecules, or an increased overall stability of the immobilised proteins. Other advantages of the described immobilization approaches are likely to include increased stability of the scFvs on the polymeric substrates due to the higher strength of binding.

CONCLUSIONS

We have described an approach for isolation, expression and modification of single-chain Fv fragments in *E. coli* for ease of targeted, covalent attachment to polymers and other solid supports. ScFvs were isolated against standard cell surface markers using a phage display approach and expressed in an *E. coli* expression platform. ScFvs were produced with C-terminal cysteine-containing linkers or glycosylation motifs for post-translation addition of a heptasaccharide. Protein modification did not affect ligand binding or, in the case of the glycosylated proteins, purified yields of the scFvs. Targeted immobilisation of the proteins on polymeric supports via their engineered C-termini resulted in significantly improved ligand

binding compared with adsorbed molecules. The described technology will facilitate creation of addition-of-function polymers with broad applications in cell targeting and capture.

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References

- [1] B. D. Ulery, L. S. Nair, C. T. Laurencin, *J. Polym. Sci. Part B: Polym. Phys.* **2011**, 49(12), 832-864.
- [2] S. Tonzani, *J. Appl. Polym. Sci.* **2013**, 129(2), 527.
- [3] A. Srivastava, I. B. O'Connor, A. Pandit, J. G. Wall, *Prog. Polym. Sci.* **2014**, 39(2): 308-329.
- [4] T. Elbayoumi, V. Torchilin, *Eur. J. Nucl. Med. Mol. Imaging* **2006**, 33(10), 1196-1205.
- [5] A. N. Lukyanov, T. A. Elbayoumi, A. R. Chakilam, V. P. Torchilin, *J. Control. Rel.* **2004**, 100(1), 135-144.
- [6] H. R. Hoogenboom, G. Winter, *J. Mol. Biol.* **1992**, 227(2), 381-388.
- [7] M. Weber, E. Bujak, A. Putelli, A. Villa, M. Matasci, L. Gualandi, T. Hemmerle, S. Wulhfard, D. Neri, *PLoS one* **2014**, 9(6):e100000.
- [8] T. J. Vaughan, A. J. Williams, K. Pritchard, J. K. Osbourn, A. R. Pope, J. C. Earnshaw, J. McCafferty, R. A. Hodits, J. Wilton, K. S. Johnson, *Nat. Biotech.* **1996**, 14(3), 309-314.
- [9] P. Pansri, N. Jaruseranee, K. Rangnoi, P. Kristensen, M. Yamabhai, *BMC Biotechnol.* **2009**, 9, 6.
- [10] A. Skerra, A. Pluckthun, *Science* **1988**, 240(4855), 1038-1041.
- [11] D. Gussow, E. S. Ward, A. D. Griffiths, P. T. Jones, G. Winter, *Cold Spring Harbor Symp. Quant. Biol.* **1989**, 54(1), 265-272.
- [12] M. Donzeau, A. Knappik, *Methods Mol Biol* **2007**, 378, 14-31.
- [13] N. Ferrer-Miralles, J. Domingo-Espin, J. L. Corchero, E. Vazquez, A. Villaverde, *Microb. Cell Fact.* **2009**, 8, 17.
- [14] R. A. Irving, A. A. Kortt, P. J. Hudson, *Immunotechnol.* **1996**, 2(2), 127-143.
- [15] P. S. Chowdhury, I. Pastan, *Nature Biotechnol.* **1999**, 17(6), 568-572.
- [16] A. Srivastava, C. Cunningham, A. Pandit, J. G. Wall, *Macromol. Biosci.* **2015**, 15(5), 682-690.
- [17] D. Y. X. Lim, Y-H. Ng, J. Lee, M. Mueller, A. B. Choo, V. V. T. Wong, *J. Biotechnol.* **2011**, 153(3-4), 77-85.
- [18] H. Lamdan, J. V. Gavilondo, Y. Munoz, A. Pupo, V. Huerta, A. Musacchio, L. Perez, M. Ayala, G. Rojas, R. F. Balint *et al.*, *Mol. bioSystems* **2013**, 9(8), 2097-2106.
- [19] A. K. Trilling, J. Beekwilder, H. Zuilhof, *Analyst* **2013**, 138(6), 1619-1627.
- [20] H-J. Um, M. Kim, S-H. Lee, J. Min, H. Kim, Y-W. Choi, Y-H. Kim, *Talanta* **2011**, 84(2), 330-334.
- [21] Z. Balevicius, A. Ramanaviciene, I. Baleviciute, A. Makaraviciute, L. Mikoliunaite, A. Ramanavicius, *Sensors Actuators B: Chemical* **2011**, 160(1), 555-562.
- [22] H. Xu, J. R. Lu, D. E. Williams, *J. Phys. Chem. B* **2006**, 110(4), 1907-1914.
- [23] J. E. Herrmann, R. M. Hendry, M. F. Collins, *J. Clin. Microbiol.* **1979**, 10(2), 210-217.
- [24] J. E. Butler, Ni, W. R. Brown, K. S. Joshi, J. Chang, B. Rosenberg, E. W. Voss, Jr, *Mol. Immunol.* **1993**, 30(13), 1165-1175.
- [25] V. Ball, P. Huetz, A. Elaissari, J. P. Cazenave, J. C. Voegel, P. Schaaf, *Proc. Natl. Acad. Sci. USA* **1994**, 91(15), 7330-7334.
- [26] R. D. Das, S. Maji, S. Das, C. RoyChaudhuri, *Appl. Surf. Sci.* **2010**, 256(20), 5867-5875.
- [27] M. J. Hortigüela, J. G. Wall, *Marine Drugs* **2013**, 11, 881-895.
- [28] X. Hu, M. J. Hortigüela, S. Robin, H. Lin, Y. Li, A. P. Moran, W. Wang, J. G. Wall, *Biomacromolecules* **2013**, 14(1), 153-159.
- [29] R. M. Horton, S. N. Ho, J. K. Pullen, H. D. Hunt, Z. Cai, L. R. Pease, in *Methods in Enzymology*, (Ed: W. Ray), Vol. 217, Academic Press, **1993**, 270-279.

- [30] L. Ge, P. Pack, C. Freund, A. Plückthun, *Expressing antibodies in Escherichia coli*, Oxford University Press, **1995**.
- [31] M. Wacker, D. Linton, P. G. Hitchen, M. Nita-Lazar, S. M. Hasla, S. J. North, M. Panico, H. R. Morris, A. Dell, B. W. Wren *et al.*, *Science* **2002**, 298(5599), 1790-1793.
- [32] J. Nielsen, N. Kulahin, P. Walmod, in *Structure and Function of the Neural Cell Adhesion Molecule NCAM*, (Ed: V. Berezin), vol. 663, Springer, New York, **2010**, 23-53.
- [33] C. Cunningham, A. Srivastava, E. Collin, S. Grad, M. Alini, A. Pandit, J. G. Wall, *PLoS one* **2013**, 8(12), e83678.
- [34] X. Hu, R. O'Dwyer, J. G. Wall, *J. Biotechnol.* **2005**, 120(1), 38-45.
- [35] F. W. Studier, *Protein Expr. Purif.* **2005**, 41(1), 207-234.
- [36] X. Hu, L. O'Hara, S. White, E. Magner, M. Kane, J. G. Wall, *Protein Expr. Purif.* **2007**, 52(1), 194-201.
- [37] Dreyer EB, Leifer D, Heng JE, McConnell JE, Gorla M, Levin LA, Barnstable CJ, Lipton SA: Proceedings of the National Academy of Sciences of the United States of America **1995**, 92(24), 11195-11199.
- [38] M. Paprocka, A. Krawczenko, D. Dus, A. Kantor, A. Carreau, C. Grillon, C. Kieda, *Cytometry Part A* **2011**, 79A(8), 594-602.
- [39] R. I. Clyman, K. A. McDonald, R. H. Kramer, *Circ. Res.* **1990**, 67(1), 175-186.
- [40] J. McCafferty, A. D. Griffiths, G. Winter, D. J. Chiswell, *Nature* **1990**, 348(6301), 552-554.
- [41] J. Hanes, A. Plückthun, *Proc. Natl. Acad. Sci. USA* **1997**, 94(10), 4937-4942.
- [42] G. Kuttner, E. Giessmann, H. Wessner, C. Scholz, D. Reinhardt, K. Winkler, U. Marx, W. Hohne, *BioTechniques* **2004**, 36(5), 864-870.
- [43] R. O'Dwyer, R. Razzaque, X. Hu, S. K. Hollingshead, J. G. Wall, *Appl. Biochem. Biotechnol.* **2009**, 159(1), 178-190.

Figure Legends

Figure 1. Schematic of antibody fragment modification and immobilization approaches.

(a) Genetically encoded tag. Additional amino acids, *e.g.* a cysteine-containing linker, are encoded in the DNA sequence. The modified protein is then immobilized on a thiol-displaying surface. (b) Post-translationally added tag. A site in the recombinant protein is modified post-translationally in the bacterial host, *e.g.* by glycosylation. After oxidation of the glycan chain, the protein is immobilized on an amine-modified surface.

Figure 2. Isolation of cell-binding antibody fragments.

(a) ELISA analysis of NCAM1-Ig1 binding of polyclonal phage-scFv populations before selection (“0”) and after 1 to 3 rounds of library panning against NCAM1-Ig1. NCAM1-Ig1 was coated on plates at 100 µg/ml. (b) Immunocytochemical binding analysis of scFvs to NCAM1-expressing rat astrocytes; left: non-binding scFv (2H12), right: F4 scFv; cell nuclei were stained with DAPI and scFvs detected using a FITC-conjugated reporter antibody; (c) FACS analysis of isotype control (left) and F4 (right) binding to rat astrocytes; (d) Screening of EPC binding of four VEGFR2-binding scFvs isolated from library screening; scFvs were detected using a FITC-conjugated anti-hexahistidine reporter; scFv 32 is in the top left panel.

Figure 3. Expression of functionalised scFv antibody fragments.

(a) Schematic of scFv expression constructs for targeted immobilisation: *ss*, bacterial signal sequence; V_H , antibody V_H domain; linker, inter-domain linker; V_L , antibody V_L domain; TEV, protease cleavage site; 6H, hexahistidine tag; Gly, glycosylation motif; Cys, cysteine-containing linker; (b) Schematic of scFv glycosylation pathway in engineered *E. coli* host cells; [31] (c) Analysis by Coomassie blue staining (left) and MAA lectin blotting (right) of purified 2H12 scFv expressed in *E. coli* cells lacking or harboring additional protein glycosylation machinery. MW: Molecular weight markers. Arrow indicates glycosylated scFv; (d) Analysis of 2H12 scFv expressed with and without cys-containing recombinant tag. MW: Molecular weight markers; RC: reducing conditions. Arrow indicates dimerised scFv.

Figure 4. Investigation of ligand binding by functionalized antibody fragments.

(a) Competitive ELISA to determine IC_{50} for fluorescein of unglycosylated (circles) and glycosylated (squares) 4M5.3 scFvs. (b) Competitive ELISA comparing domoic acid binding of unmodified 2H12 scFv (empty circles) and cysteine-tagged 2H12 scFv (clear triangles) adsorbed on polystyrene plates, as well as cysteine-tagged 2H12 scFv covalently immobilised on a maleimide-activated plate (filled triangles). (c) Unmodified (left) and glycosylated (right) scFv 32 binding to endothelial progenitor cells.

Figure 5. Binding activities of polymer-linked scFvs.

(a) Domoic acid binding of anti-domoic acid scFv fragments immobilised on maleimide-activated polystyrene plates. Right: detection of immobilized scFvs. Left: domoic acid binding of immobilized scFvs. Solid columns: unmodified 2H12 scFv; hatched columns: cys-tagged scFvs. (b) Fluorescein binding of 4M5.3 scFvs immobilized on EDA-agarose via ionic adsorption (triangles) or via their C-terminal glycan tag (squares).

Figure 1

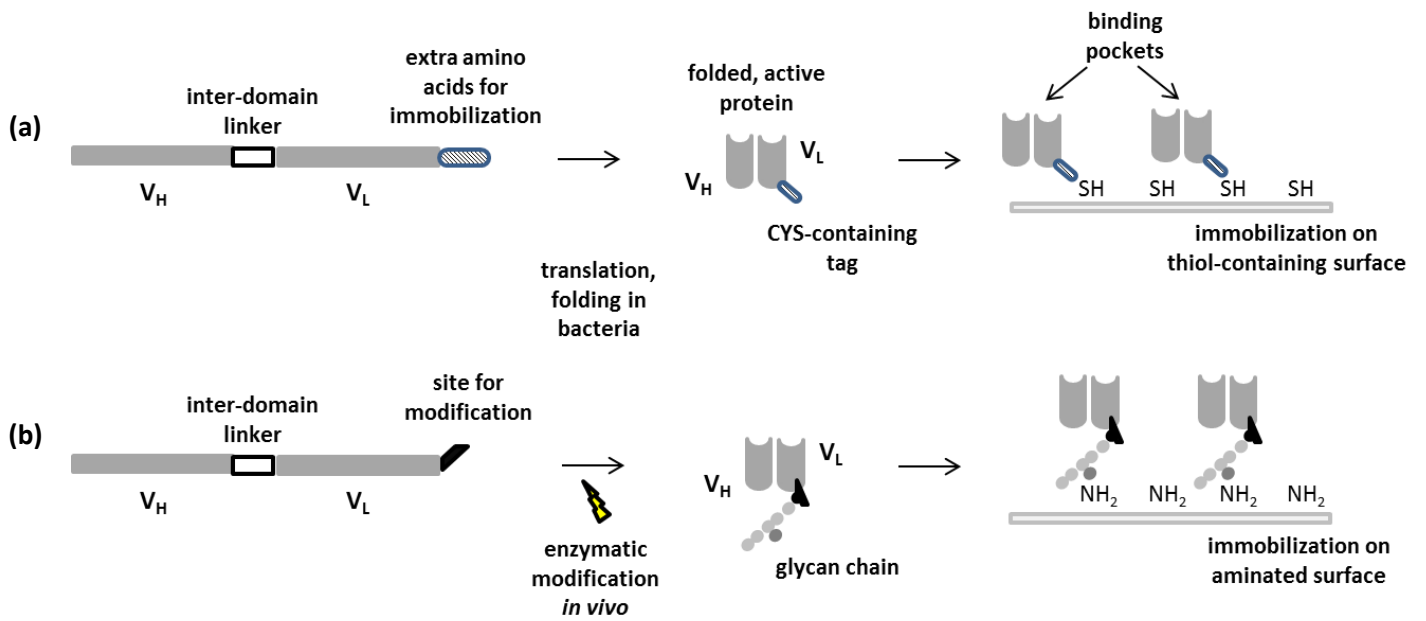
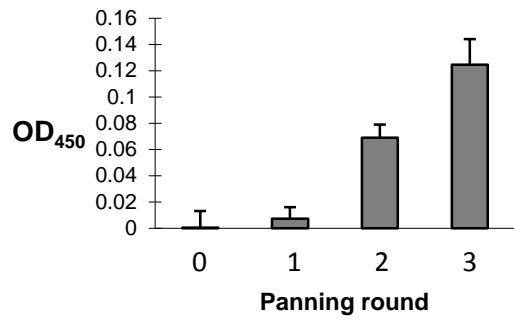
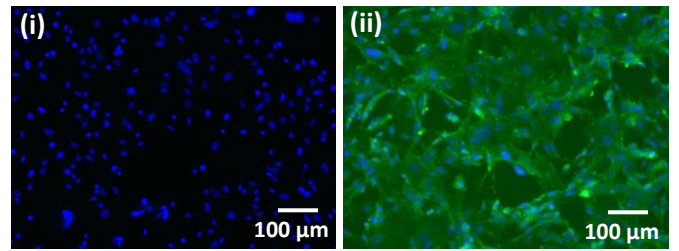


Figure 2

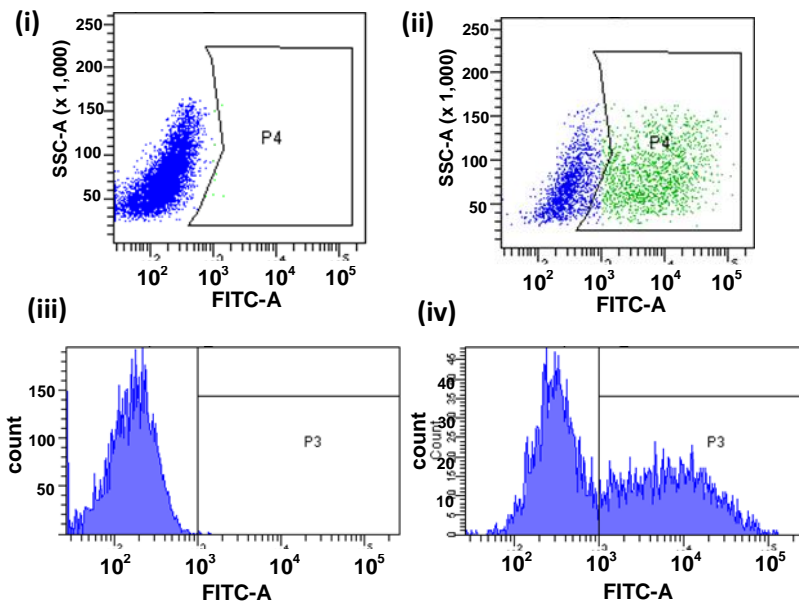
(a)



(b)



(c)



(d)

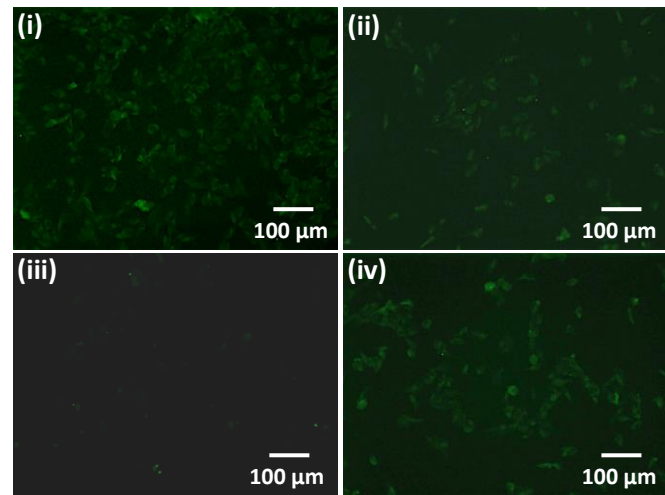


Figure 3

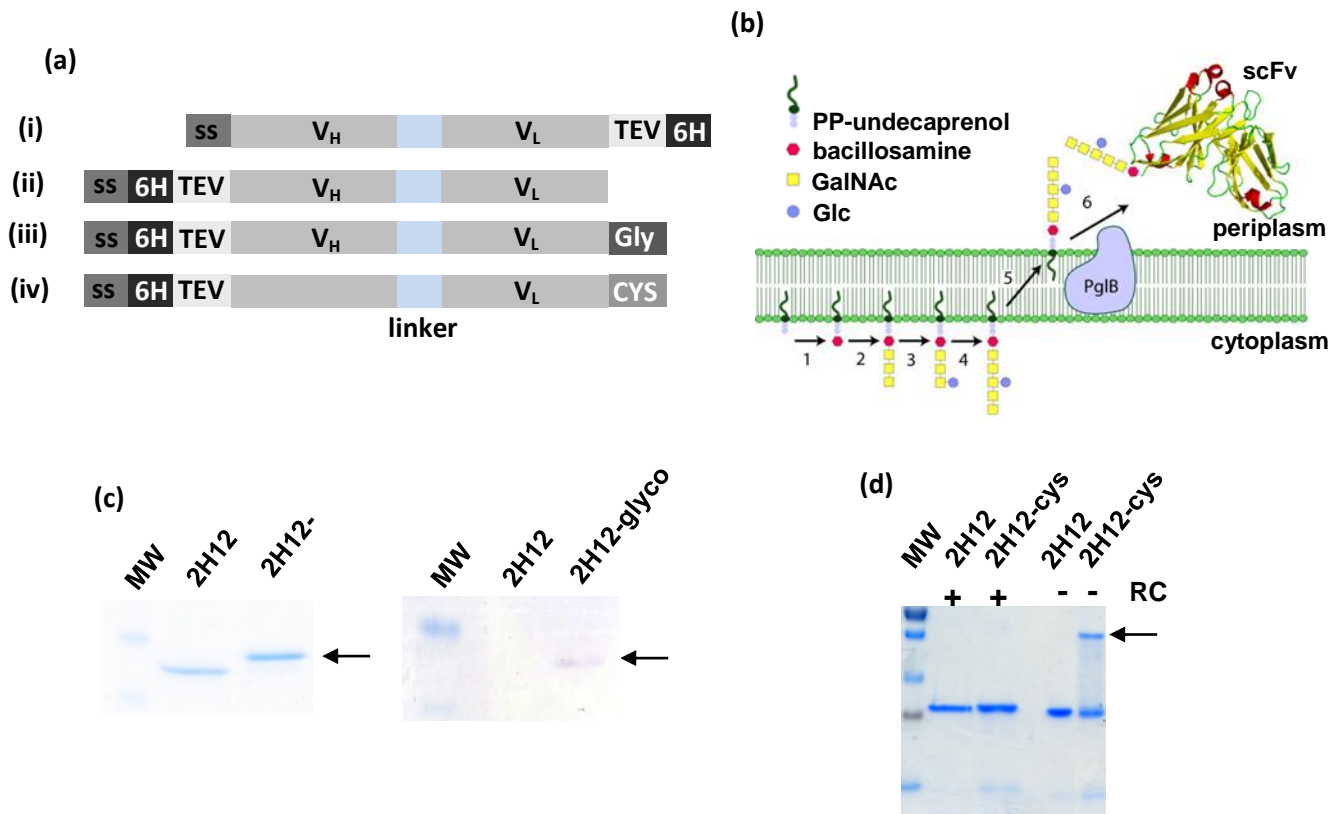


Figure 4

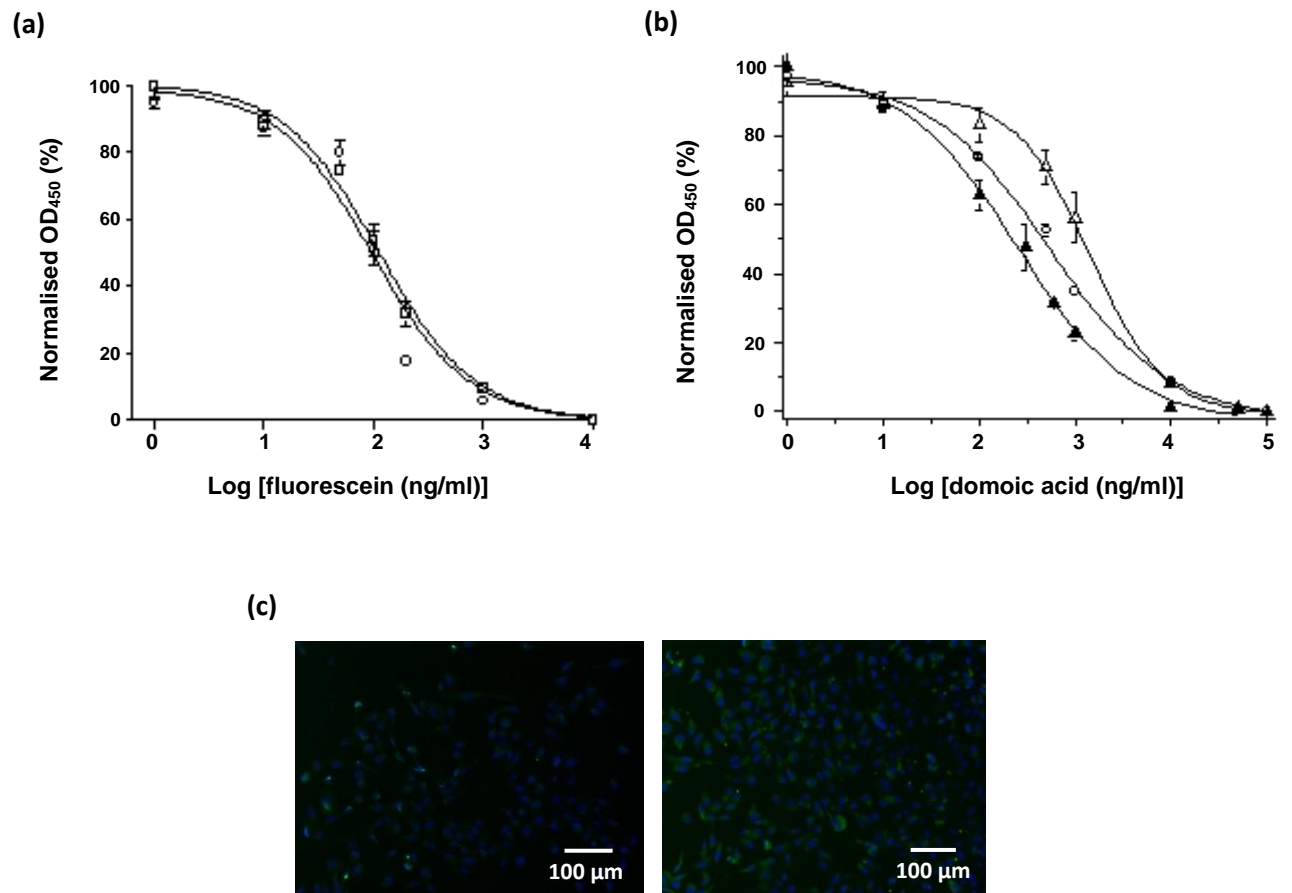


Figure 5

