ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Engineering of an Anti-Epidermal Growth Factor Receptor Antibody to Single Chain Format and Labeling by Sortase A-Mediated Protein Ligation

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ABSTRACT: Sortase-mediated protein ligation is a biological covalent conjugation system developed from the enzymatic cell wall display mechanism found in *Staphylococcus aureus*. This three-component system requires: (i) purified Sortase A (SrtA) enzyme; (ii) a substrate containing the LPXTG peptide recognition sequence; and (iii) an oligoglycine acceptor molecule. We describe cloning of the single-chain antibody sc528, which binds to the extracellular domain of the epidermal growth factor receptor (EGFR), from the parental monoclonal antibody and incorporation of a LPETGG tag sequence. Utilizing recombinant SrtA, we demonstrate successful incorporation of biotin from GGG-biotin onto sc528. EGFR is an important cancer target and is over-expressed in human tumor tissues and cancer lines, such as the A431 epithelial carcinoma cells. SrtA-biotinylated sc528 specifically bound EGFR expressed on A431 cells, but not negative control lines. Similarly, when sc528 was labeled with fluorescein we observed antigen-specific labeling. The ability to introduce functionality into recombinant antibodies in a controlled, site-specific manner has applications in experimental, diagnostic, and potentially clinical settings. For example, we demonstrate addition of all three reaction components in situ within a biosensor flow cell, resulting in oriented covalent capture and presentation of sc528, and determination of precise affinities for the antibody–receptor interaction.

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KEYWORDS: scFv; bioconjugation; EGFR; enzyme-mediated ligation; biosensor

Introduction

Gram positive bacteria, including pathogens, such as *Staphylococcus aureus*, *Bacillus anthracis*, and *Streptococcus pyogenes*, have evolved a sophisticated generic system to achieve covalent attachment of proteins including virulence factors to their cell wall, utilizing the so-called Sortase family of enzymes (Hendrickx et al., 2011; Marraffini et al., 2006). Sortase A (SrtA) from *S. aureus* was the first of these enzymes to be described, and is itself an integral membrane protein attached to the outer surface of the bacterial cell (Hendrickx et al., 2011). There, it accepts proteins exported through the Sec secretory apparatus which are tagged with an ^{N}LPETG^{C} (or equivalent) motif. Through an incompletely understood enzymatic process, the sortase covalently couples the C-terminus to the free N-terminus of polyglycine acceptor peptides located on the cell wall (Suree et al., 2009; Weiner et al., 2010). This elegant solution to coupling a significant number of proteins through the one mechanism resulted in SrtA being regarded as an essential housekeeping enzyme and attractive antimicrobial drug target (Suree et al., 2007). Subsequently, further sortase variants have been identified, often with specialized functions, such as pilus growth and attachment (Paterson and Mitchell, 2004), and an additional level of complexity appears to operate through the sequential activity of different sortase types in the staged assembly of multi-domain proteins (Kang et al., 2009).

The discovery of this naturally selected and robust protein ligation system (evolved to operate in the hostile extracellular milieu encountered during bacterial host invasion) has been exploited to deliver a variety of applications for protein engineering (Mao et al., 2004; Proft, 2010; Tsukiji and Nagamune, 2009). Examples of the in vitro use of sortase-mediated ligation include protein PEGylation (Popp et al., 2011); attachment to solid supports, such as polystyrene beads (Parthasarathy et al., 2007), introduction of novel functionality (Proft, 2010), fluorescent labeling of cell surface proteins (Yamamoto and Nagamune, 2009), and novel protein purification technologies utilizing SrtA as a solubility tag (Caswell et al., 2010).

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The epidermal growth factor receptor (EGFR) family consists of four related transmembrane proteins: EGFR/HER/ErbB1, HER2/Neu/ErbB2, HER3/ErbB3, and HER4/ErbB4 (Burgess and Thumwood, 1994; Elleman et al., 2001). EGFR binds and is activated by epidermal growth factor (EGF), as well as a number of other important growth factors, such as transforming growth factor-α (TGFα; Garrett et al., 2002) and is widely expressed in human tissues, where its main biological functions include mediation of cell growth and survival, cell development, and differentiation. Consequently, EGFR over-expression on the cell surface and activation is a feature of many human cancers (Lewis Phillips et al., 2008; Rossi et al., 2006) including tumors of breast, brain, head & neck, and endometrium. As such, the EGFR has emerged as a major therapeutic target, with both small molecule inhibitors of the receptor tyrosine kinase domain, and chimeric and humanized monoclonal antibodies (mAbs) that bind the EGFR extracellular domain, blocking ligand binding/receptor activation, currently in clinical use.

The EGFR-targeting antibody, 528, is a mouse IgG mAb (Kawamoto et al., 1983; Sato et al., 1983) which binds domain III of the human EGFR, with high affinity, competing with EGF family ligands for receptor binding (Kawamoto et al., 1983). It also inhibits the proliferation in vitro of a variety of cultured malignant human cell lines expressing EGFR, and in combination with cisplatin produces striking anti-tumor effects in well-established tumor xenografts (Fan et al., 1993). A clinical counterpart of mAb 528, a chimeric human-mouse antibody called Erbitux (cetuximab, C225; Kim et al., 2001) is approved for metastatic colorectal cancer (Jonker et al., 2007) and head and neck cancers (Herbst et al., 2001). The variable regions have also been humanized (Makabe et al., 2008).

Chemical and biological conjugation of small molecule cytotoxic drugs or imaging agents to mAbs and their recombinant fragments is both an established and expanding area of study, combining the specificity of antibody-mediated tissue or tumor targeting with the delivery of small molecule cytotoxic drugs and/or imaging agents (Carter and Senter, 2008; Chari, 2008; Ducry and Stump, 2010; Senter, 2009). One major drawback with many examples of this technology has been the inability to precisely conjugate drug and imaging agents to specific residues/domains of interest on the antibody molecule without impacting on antibody activity including pharmacokinetics (Alley et al., 2010). Here, we describe the cloning and recombinant production of the single chain Fv fragment of mAb 528 (sc528; Fig. 1A). We utilize sortase-mediated protein ligation to accomplish site-specific covalent attachment of functional molecules to the C-terminus of this scFv, and demonstrate the targeting of EGFR-expressing mammalian cell lines and downstream applications in biosensor and kinetic analysis.

Materials and Methods

Cloning, Expression, and Purification of SrtA

A DNA cassette encoding residues 60–208 of the *S. aureus* SrtA was synthesized at Geneart AG (Regensburg, Germany, www.geneart.com) in the expression vector pET28a (Novagen). The sequence included 5′ *Nde*I and 3′ *Bam*H1 sites, and initiating methionine and N-terminal hexahistidine tag sequences (Supp Fig. S1). Details of pET28a–SrtA protein expression are described in the Supplementary materials.

mRNA Extraction, PCR, Cloning and Sequencing

The 528 murine hybridoma cell line (IgG1/kappa isotype) was utilized for PCR amplification of variable heavy (V_H) and variable light (V_L) chain cDNA. Total RNA was extracted from $\sim 5 \times 10^6$ cells using TRIzol reagent (Invitrogen, VIC, Australia) and messenger RNA subsequently isolated using the Oligotex mRNA kit (Qiagen Pty Ltd, VIC, Australia). The V_H and V_L cDNAs were synthesized according to previously described methodology (Gilliland et al., 1996). Resulting PCR products were ligated

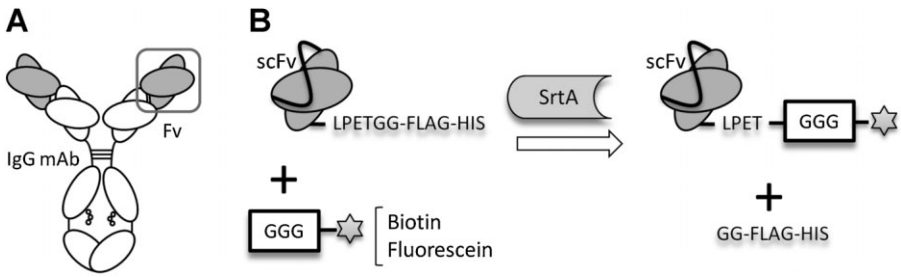

Figure 1. **A**: The mature IgG immunoglobulin is a homodimer of paired heavy and light chains. Antigen binding is encapsulated in the Fv fragments, each consisting of variable heavy (V_H) and variable light (V_L) domains. **B**: Scheme for SrtA-mediated protein ligation. The single chain Fv fragment, a single polypeptide chain, is enzymatically ligated to a polyglycine construct (biotin or fluorescein), resulting in liberation of downstream amino acid residues (FLAG-HIS affinity tags), and C-terminal covalent attachment of the labeling group to the recombinant antibody.

into pPCR-Script Amp SK (+) vector (Stratagene La Jolla, CA), sequenced, and analyzed for consensus V_H and V_L sequences.

For scFv construction the sc528 gene fragment was assembled by the Splice Overlap Extension (SOE) PCR method. Specifically, V_H and V_L genes were first amplified using S528VHNcoI (GCCCAGCCGGCCATGGCTcaggt ccaactgcagcaacc) and as528VH (accggaaccgccgccaccggatccgc cgccgcctgaggagactgtgagactggtgcc) primers for V_H; and s528VL (TCCGGTGGCGGCGGTTCCGGTGGTGGTGGG TCAgatgttttgatgacccaaactcc) and as528VLNot (gtggtgctcgag tgcggccgcccgttttatttccagcttggtc) primers for V_L. Resulting gene fragments of ~350 bp containing overlapping and complementary linker sequences at the 3′ end of V_H and 5′ end of V_L were assembled into an scFv fragment by SOE PCR, giving an ~700 bp scFv DNA fragment. Next, the sortase recognition sequence was added to the 3′ end with C-terminal primer as528Sort (ataatctgcggccgcaccgccggtttcc ggcagccgttttatttccagcttggtcc). The sc528 fragment was cloned into an "in-house" *Escherichia coli* expression vector pGC at *Nco*I and *Not*I restriction sites. The pGC vector is a pUC19 derivative, incorporating an N-terminal cleavable *pel*B periplasmic targeting signal and C-terminal FLAG and hexahistidine peptide epitopes for affinity purification. Ligations were transformed into *E. coli* TG1 cells, and putative positive colonies verified by DNA sequencing.

Synthesis and Purification of Functionalized Poly-Glycine Molecules

Synthesis of all conjugates is described in the Supplementary Materials and Supp Figure S2.

Expression, Purification and Analysis of sc528

Expression and purification of sc528 was performed as previously described (Nuttall et al., 2004). Briefly, *E. coli* TG1 starter cultures were grown overnight in 2YT medium (ampicillin 100 μg/mL/glucose, 2.0% w/v), diluted 1/100 into fresh 2YT (100 μg/mL ampicillin/glucose, 0.1% w/v), and then grown at 37°C/200 rpm until $A_{550\,nm} = 0.7$. Cultures were then induced with IPTG (1 mM final), grown for a further 5 h at 24°C, and harvested by centrifugation (Beckman JA-14/5, 524 g/15 min/4°C). Periplasmic fractions were isolated by the method of Minsky (Minsky et al., 1986) and recombinant protein purified by affinity chromatography using an anti-FLAG antibody-Sepharose column (10 cm × 1 cm). The affinity column was equilibrated in TBS, pH 7.4, and bound protein eluted with 0.1 M glycine, pH 3.0. Following FLAG affinity purification, protein peaks corresponding to scFv monomer fraction were further purified by size-exclusion chromatography on a Superdex 200 10/300 GL (GE Healthcare, VIC, Australia) equilibrated in TBS (PBS was not used as the presence of phosphate chelates calcium, an essential component of the SrtA reaction buffer). The column was eluted at 0.5 mL/min and 1 mL fractions collected. The sc528 protein was stored as aliquots at 4°C prior to use. Recombinant proteins were analyzed by non-reducing SDS–PAGE through 12% Tris/glycine gels.

Sortase-Mediated sc528 Labeling

A solution containing 20 μM sc528, 2 mM oligoglycine biotin, and 60 μM SrtA was incubated for 3 h at 37°C. The reaction was performed in SrtA reaction buffer (50 mM Tris, 150 mM NaCl, 10 mM $CaCl_2$, pH 7.5). The final volume of reaction mixture was 200 μL. The biotinylated sc528 was purified from the reaction mixture using small-scale His-tag pull-down purification (Ni-NTA superflow; Qiagen) and was stored in small aliquots at 4°C prior to use.

ELISA Assays

Recombinant EGFR ectodomain (sEGFR-501; Elleman et al., 2001; Garrett et al., 2002) or negative control protein antigens (0.5 μg/well in PBS) were coated onto Maxisorb Immuno-plates (Nunc, Germany) and incubated at 4°C overnight. Plates were rinsed three times with washing buffer (1× PBS containing 0.05% Tween20), blocked with 5% BSA in PBS for 2 h at RT, and incubated with biotinylated sc528 for 1 h at RT. Plates were washed three times with washing buffer, and streptavidin conjugated with horse radish peroxidise (HRP; 1/3,000 dilution in PBS/2% BSA; Pierce, Illinois), or appropriate anti-hexahistidine-HRP or goat anti-mouse-HRP control secondary antibody was added. ELISAs were developed using ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; Boehringer, Mannheim, Germany), and read after 15 min in an ELISA reader (Organon Teknika Reader 230S) at $A_{405\,nm}$.

Cell-Based ELISA Assays

A431 cells were maintained as monolayers at 37°C in a 5% CO_2 atmosphere in DMEM (Invitrogen, Victoria, Australia) supplemented with 10% (v/v) FCS (JRH Biosciences, Missouri) and 1% glutamax (Invitrogen). Twenty-four hours prior to ELISA analysis, cells were seeded in a 96-well plate in complete medium so that they were at confluency the next day. Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Pennsylvania) for 15 min then blocked with blocking buffer (10% FCS; JRH Biosciences) and 2% BSA in 1× PBS) for 2 h. Cells were then labeled with biotinylated sc528 and unlabeled sc528 in blocking buffer for 1 h. Cells were washed three times with 1× PBS then incubated with an appropriate secondary antibody conjugated with HRP in blocking buffer for 1 h, before developing as above.

Cell-Based Immunofluorescence Assays

A431 cells were plated in complete medium on coverslips (ProSciTech, coverglass no. 1, Ø 13 mm) coated with poly-L-lysine (Sigma–Aldrich, Missouri) and incubated at 37°C until cells reached 50% confluence. Cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences) for 15 min, then blocked with blocking buffer (10% FCS in 1× PBS containing 2% BSA) for 2 h. Cells were then labeled with biotinylated sc528, unlabeled sc528, and sc528 labeled with fluorescein overnight at 4°C. Cells were washed three times with 1× PBS then incubated with secondary antibody in blocking buffer for 1.5 h. Cells were washed ten times with 1× PBS then stained with DAPI (1 μg/mL; Sigma–Aldrich) for 15 min. Cells were mounted using fluorescent mounting medium (Dako, Victoria, Australia) and visualized using a fluorescence microscope (Olympus BX60, NSW, Australia).

Surface Plasmon Resonance

Binding kinetics of the interaction between sc528 and sEGFR-501 were determined by SPR in a Biacore T100 instrument (GE Healthcare). The sc528 was immobilized onto a CM5 chip (GE Healthcare) surface by SrtA-mediated ligation via a pre-immobilized Gly3-linker peptide ($^{NH2}GGGSSC_{COOH}$; Auspep, Australia). Unless otherwise stated, all immobilizations and binding experiments were performed at 25°C with 1× HBS-P (10 mM HEPES pH 7.4, 150 mM NaCl, 0.005% Tween-20) as instrument running buffer. Approximately 450 RU (1 RU = 1 pg of protein/mm^2) of Gly3-linker peptide was "thiol-coupled" in a single flow cell on the chip surface using the previously described coupling protocol by Clow et al. (2008). Biacore T100 analysis temperature was adjusted to 37°C and the instrument primed into 1× TBS-P+/2 mM $CaCl_2$ buffer. Sortase mediated coupling of sc528 was achieved by injecting a SrtA reaction mixture (consisting of 10 μM sc528, 15 μM SrtA in sortase reaction buffer) over the immobilized Gly3-linker peptide at 1 μL/min for 1 h at 37°C. Due to minor non-specific binding of reaction mix (presumably SrtA) to the chip surface it was not possible to accurately determine the covalently-coupled amount (RU) of sc528. To remove non-covalently bound material, the chip surface was washed by repeated 1-min injections of 1 M NaCl at 20 μL/min until the baseline steadied. For control purposes, the sc528 was also immobilized in a separate flow-cell on the same CM5 chip using a standard protocol for coupling of proteins via primary amines to a carboxymethyldextran-modified chip surface(Johnsson et al., 1991). Approximately 130 RU of sc528 was amine-coupled to the chip surface. To perform binding analysis of the two differently immobilized sc528 proteins, recombinant sEGFR-501 protein concentrations were prepared by a threefold dilution in 1× HBS-P buffer from 81 to 1 nM and injected sequentially over the chip surface at a flow rate of 30 μL/min and contact time of 180-s and a dissociation time of 300-s. The surface was regenerated between each binding cycle with a 10-s injection of 36 mM phosphoric acid at 30 μL/min. Binding data were processed and analyzed using Scrubber software (www.biologic.com.au). To determine the kinetic rate constants of binding interactions, processed data were fit globally to a simple 1:1 interaction model that included mass transport component. The ratio of the rate constants (k_d/k_a) yielded the value for the equilibrium dissociation constant (K_D).

Results

Sortase-Mediated Protein Ligation—A Three-Component System

Three components are required for this protein ligation reaction: (i) purified SrtA enzyme; (ii) a substrate containing the LPETG sortase recognition sequence (SRT); and (iii) an oligo-glycine acceptor molecule (Fig. 1B). The coding sequence for the *S. aureaus* SrtA Δ59 variant, which lacks the N-terminal membrane anchor sequence, was chemically synthesized and cloned into *E. coli* expression vector pET28a, downstream of a hexahistidine affinity tag. Recombinant enzyme (~18.9 kDa) was readily purified by metal ion affinity chromatography followed by gel filtration chromatography, and was routinely concentrated to ~500 μM (Supp Fig. S1). SrtA identity was validated by mass spectrometry and N-terminal protein sequencing, and activity confirmed in model systems prior to use.

The mAb 528 was cloned from the parental monoclonal cell line and converted to single chain format by sequential polymerase chain reaction (PCR) amplification of the V_H and V_L chain sequences. Further splice-overlap PCR and cloning into *E. coli* expression vector pGC was used to produce a final DNA construct (sc528) encoding an ~29.4 kDa protein, including the 15 residue glycine–serine linker connecting the two antibody variable domains into the scFv format (Fig. 2A). The SrtA recognition sequence (LPETGG), and sequential FLAG and hexahistidine tag sequences (for affinity purification purposes) were located at the C-terminus and in a position normally occupied by the hinge region to the mature antibody constant regions (Fig. 1A). This distanced the SrtA-cleaved sequential FLAG and hexahistidine tags from the antigen-binding site (paratope), significantly reducing the possibility of interference with the antibody–antigen interaction. Like many scFv antibody fragments, only modest yields of sc528 were obtained from the *E. coli* periplasmic space (~6 mg/L) following FLAG affinity purification, however, the majority of this protein was in the preferred monomeric fraction by gel filtration (Fig. 2B). Again, the identity and activity of the protein was confirmed by SDS–PAGE (Fig. 2C), N-terminal sequencing, mass spectrometry, and ELISA assay utilizing immobilized EGFR protein (results not shown). The third components of the system, functionalized oligo-glycine constructs, were prepared synthetically (see Supplementary Materials and Supp Fig. S2). Two variants, Gly3-fluorescein

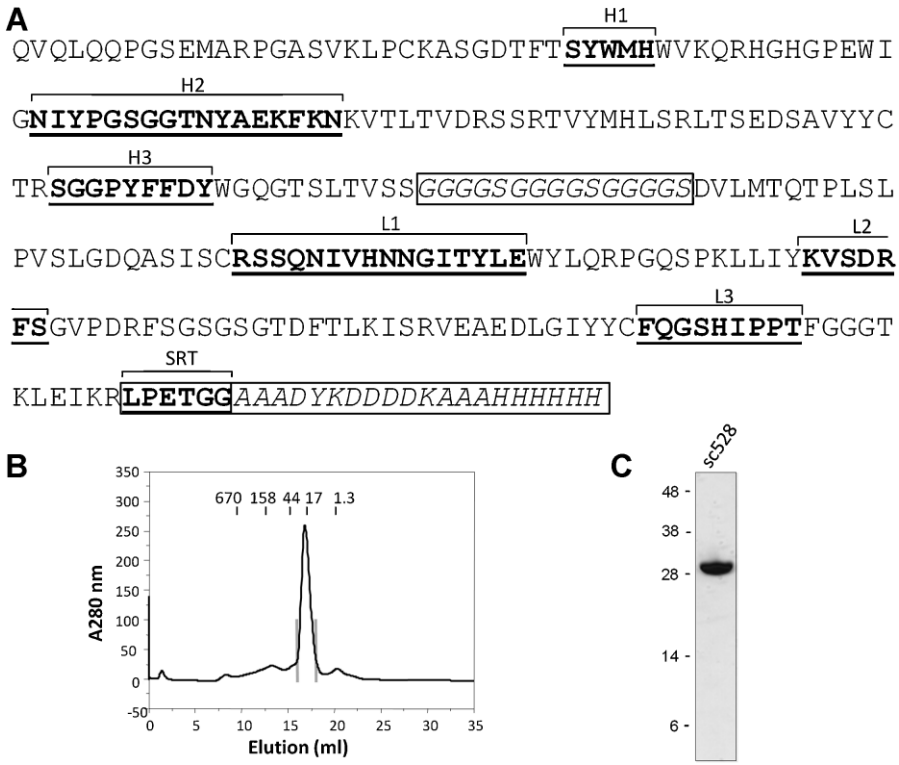

Figure 2. Cloning and expression of sc528. **A**: The sc528 is a single polypeptide chain of ~29.4 kDa. Complementarity determining region (CDR) loops are indicated for the variable heavy (H1, H2, H3) and variable light (L1, L2, L3) antibody chains. The 15 residue V_H–V_L linker, C-terminal FLAG and hexahistidine tags, and the Sortase recognition sequence (SRT) are boxed. **B**: Analysis on Superdex 200 10/300 GL of affinity-purified sc528. Vertical gray lines indicate the monomeric protein fraction collected for conjugation experiments. Elution times for standard proteins (kDa) are also shown. **C**: SDS–PAGE of purified sc528 from (B). Approximated molecular masses (kDa) are given to the left.

and Gly3-biotin (fluorescein-GGG; biotin-GGG) were isolated in high purity and characterized by ^{1}H NMR, ES-MS, and analytical HPLC.

SrtA-Mediated sc528 Biotinylation

In an initial set of experiments, sc528 was biotinylated using SrtA and biotin-GGG. This reaction was monitored by two approaches. First, with a mixture of all three components listed above, a single band of ~27 kDa was observed by SDS–PAGE, consistent with addition of biotin to the intact scFv protein and loss of the FLAG-hexahistidine affinity tags (Fig. 3A). This band co-migrated with the SrtA, but could clearly be distinguished by transfer to a solid support (Western blot) and probing with a streptavidin-HRP conjugate (Fig. 3B). Following conjugation, no FLAG-reactive residual scFv was observed, indicating efficient cleavage of the tag sequences from the C-terminus to the SrtA recognition site (results not shown). In these experiments, high molecular weight intermediates were also present, representing (i) dimers of the SrtA enzyme (Lu et al., 2007); and (ii) an intermediate product probably representing a reaction intermediate containing SrtA and the sc528-LEPTG. The presence of such composites is a feature of the system in our hands, and the reaction can be driven closer to completion by the addition of excess poly-glycine substrate (M. Madej, unpublished data). Secondly, products from the biotinylation reaction of sc528, were tested for binding to sEGFR-501 by a Streptavidin ELISA assay, where the biotin-labeled sc528 antibody specifically recognized sEGFR-501 but did not respond to a series of negative control antigens (Fig. 3C). Thus, we can conclude that sortase-mediated biotinylation did not interfere with either antibody specificity or antigen binding. No ELISA response was observed for assays containing either unconjugated sc528, or in the absence of secondary antibody (Supp Fig. S3).

Sortase-Biotinylated sc528 Recognizes EGFR on A431 Cells

To demonstrate that sc528 biotinylated by SrtA-mediated ligation recognized native EGFR, a series of cell-based ELISA assays were performed utilizing the A431 epidermoid carcinoma cell line, which expresses $1–3 \times 10^6$ EGFR molecules per cell (Kawamoto et al., 1983). Biotinylated

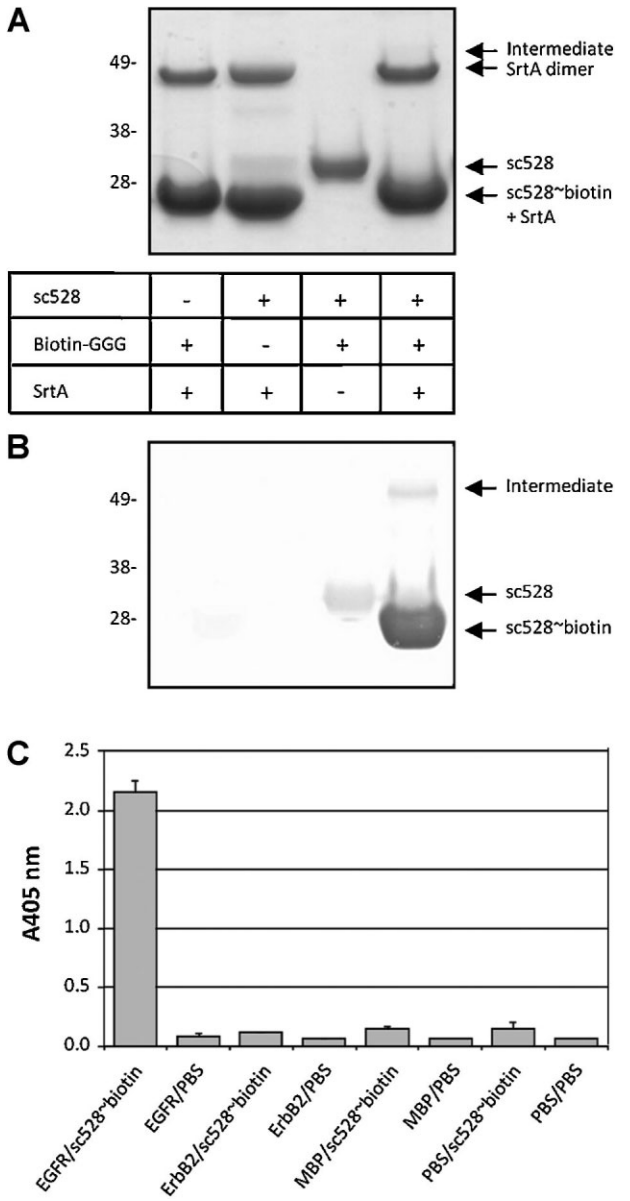

Figure 3. SrtA-mediated sc528 biotinylation. **A**: SDS–PAGE reveals a decrease in scFv antibody molecular mass in the presence of all three reaction components. **B**: As for (A) except Western blot probed with streptavidin-HRP. Biotinylated scFv antibody is present for all three components. An intermediate product consisting of SrtA and sc528 is also faintly visible, as well as some non-specific immune-decoration of the unreacted scFv (lane 3). **C**: Binding of biotinylated sc528 (1.5 μM) to EGFR and negative control antigens (0.65 μM), including the related receptor tyrosine kinase ErbB2 and the maltose-binding protein (MBP). The assay was performed in fourfold and signals were plotted as average optical readings of the replicates ±SD.

sc528 reacted strongly with fixed cells when detected with a HRP-conjugated secondary antibody, but not when detected with non-biotinylated sc528 or in the absence of primary or secondary antibodies (Fig. 4). This specific cell-surface labeling was further illustrated by confocal microscopy of A431 cells utilizing an Alexa 488-Streptavidin conjugate. Clear patterns of strong cellular fluorescence were observed

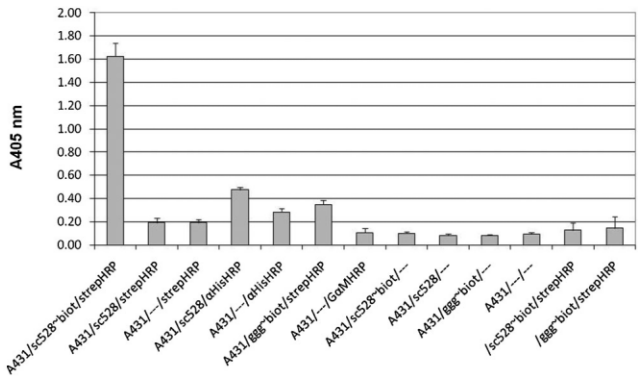

Figure 4. SrtA-biotinylated sc528 recognizes A431 EGFR over-expressing cells. A431 cells were seeded in a 96-well plate format, fixed, and tested for binding of biotinylated sc528, and unlabeled sc528. Varying primary and secondary antibodies were omitted in a series of controls. Results represent the average ± SD of triplicate wells.

for SrtA-biotinylated sc528 (Fig. 5, panel A), but not for non-biotinylated sc528, or in the absence of primary or secondary antibody (Fig. 5, panels B–D). This pattern of labeling parallels that observed for control anti-EFGR mAbs (results not shown) and is indicative of surface receptor labeling.

Direct Fluorescein Labeling of sc528

In order to eliminate the requirement for a labeled secondary reagent (HRP-conjugated streptavidin) in our system, we next attempted to directly label sc528 utilizing poly-glycine fluorescein. This reaction was as efficient as the biotinylation reaction, however low product is observed by protein staining (Fig. 6A). However, a fluorescently labeled product was clearly observed upon transfer to solid support, indicating successful SrtA-mediated ligation of the fluorescent dye to the scFv (Fig. 6B). Addition of sc528-fluorescein to A431 cells over-expressing EGFR again resulted in clearly detectable and specific cell-surface labeling, but at a level below successful photographic capture due to fluorescence quenching (Harris and Mutz, 2006).

Sortase-Mediated Conjugation of sc528 to a Biosensor Surface

The ability to introduce additional functionality into recombinant antibodies in a controlled, site-specific manner has potential application in diagnostic and kinetic determination settings. To illustrate this concept we performed a series of biosensor binding experiments utilizing sc528. A CM5 chip was functionalized by attachment, in this case a Gly3-SSC peptide through thiol linkage to the 2-(pyridinyldithio)ethane amine (PDEA) activated carboxymethyl dextran surface (Fig. 7A). Slow co-injection of SrtA and

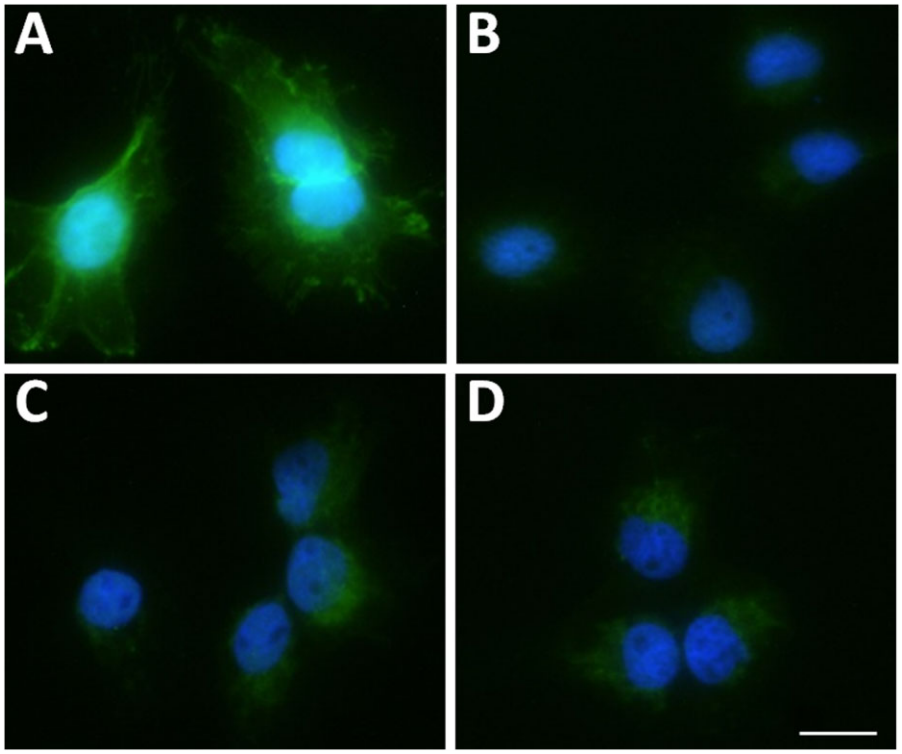

Figure 5. Immunofluorescent labeling of EGFR on A431 cells. Non-permeabilized A431 cells were labeled with (**A**) sc528-biotin, detected with Streptavidin Alexa 488; **B**: sc528, detected with Streptavidin Alexa 488; **C**: Streptavidin Alexa 488 alone; and **D**: GGG-biotin, detected with Streptavidin Alexa 488 and visualized using a fluorescence microscope at 488 nm. Sc528-biotin specifically recognizes EGFR on the surface of A431 cells. Scale bar 20 μm.

sc528 (at 1 μL/min) allowed for in situ (with the chip docked in the instrument) sortase-mediated coupling of the sc528 to the Gly3-SSC acceptor peptide. This methodology generated a protein surface where all the antibody molecules were conjugated through their C-termini, resulting in the antigen-binding paratope being immobilized in a homogeneous and oriented manner. This is in contrast to standard amine-mediated coupling where the immobilized antibody is bound mainly through lysine side chains and therefore randomly oriented on the chip surface. A series of kinetic experiments with sortase-conjugated sc528, utilizing sEGFR-501 as analyte, were performed and fit a 1:1 binding model (Fig. 7B) yielding an affinity of ~3 nM ($k_a = 2.08 \pm 0.02 \times 10^5\,M^{-1}\,s^{-1}$; $k_d = 6.1 \pm 0.1 \times 10^{-4}\,s^{-1}$; $K_D = 2.94 \pm 0.08 \times 10^{-9}\,M$). This compared well with affinities obtained for the amine-coupled sc528 ($k_a = 1.47 \pm 0.01 \times 10^5\,M^{-1}\,s^{-1}$; $k_d = 4.85 \pm 0.02 \times 10^{-4}\,s^{-1}$; $K_D = 3.30 \pm 0.02 \times 10^{-9}\,M$), again indicating that C-terminal coupling (and immobilization) did not adversely impact upon antibody affinity. These affinity estimates were also similar to those obtained for the parental mAb 528 immobilized to the biosensor surface ($K_D = 2.1 \times 10^{-9}\,M$; Hong et al., 2011) where sEGFR-501 was used as analyte, indicating that conversion to scFv format had not compromised antibody activity.

Discussion

This work demonstrates the use of SrtA to promote site-specific ligation of small molecule GGG constructs to the therapeutically relevant sc528 scFv without compromising a key determinant of antibody function—the ability to bind antigen with high affinity. This is consistent with a recent study that successfully employed SrtA-mediated ligation to conjugate full-length IgGs, either through a triglycine labeled N-terminus on both the heavy and light chain, or via an LPETG C-terminal light chain tag, with a number of proteins including green fluorescent protein (GFP) and a fragment of the A33 cell-surface antigen, without compromising the functionality of either conjugate pair (Levary et al., 2011). Similarly, Ta et al. (2011) reported SrtA-mediated scFv ligation to a reporter protein, contrast agents, and cells, for in vivo targeting of glycoproteins expressed on activated platelet. These studies, combined with our results, clearly demonstrate the broad utility of this technology in producing biologically active antibody-based imaging and targeting conjugates.

A number of very recent examples have highlighted further biotechnological applications in sortase-mediated functionalization of therapeutic proteins and peptides. For example, SrtA-directed, site-specific PEGylation of several

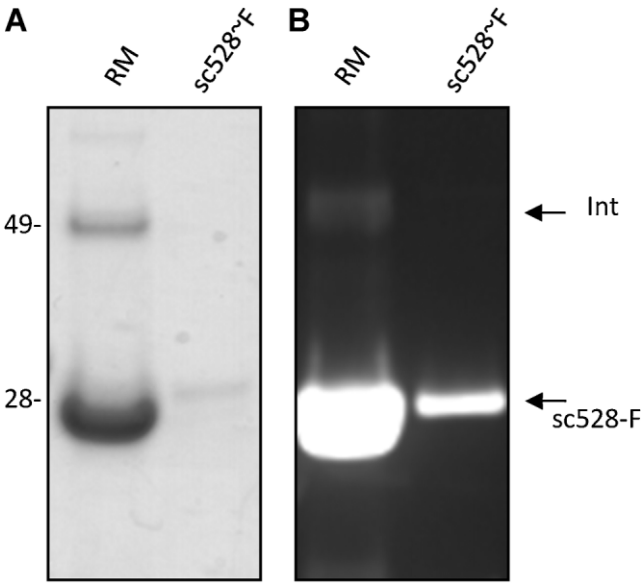

Figure 6. Direct fluorescent labeling of sc528. **A**: SDS–PAGE analysis of SrtA-mediated conjugation of GGG-fluorescein to sc528. The gel shows the unpurified reaction mixture (RM), and antibody fraction (sc528∼F) following removal of SrtA and un-reacted reagents. **B**: As for (A), following transfer to solid support by Western blot and visualization at 494 nm. An intermediate product (Int; described in Fig. 3) is also apparent.

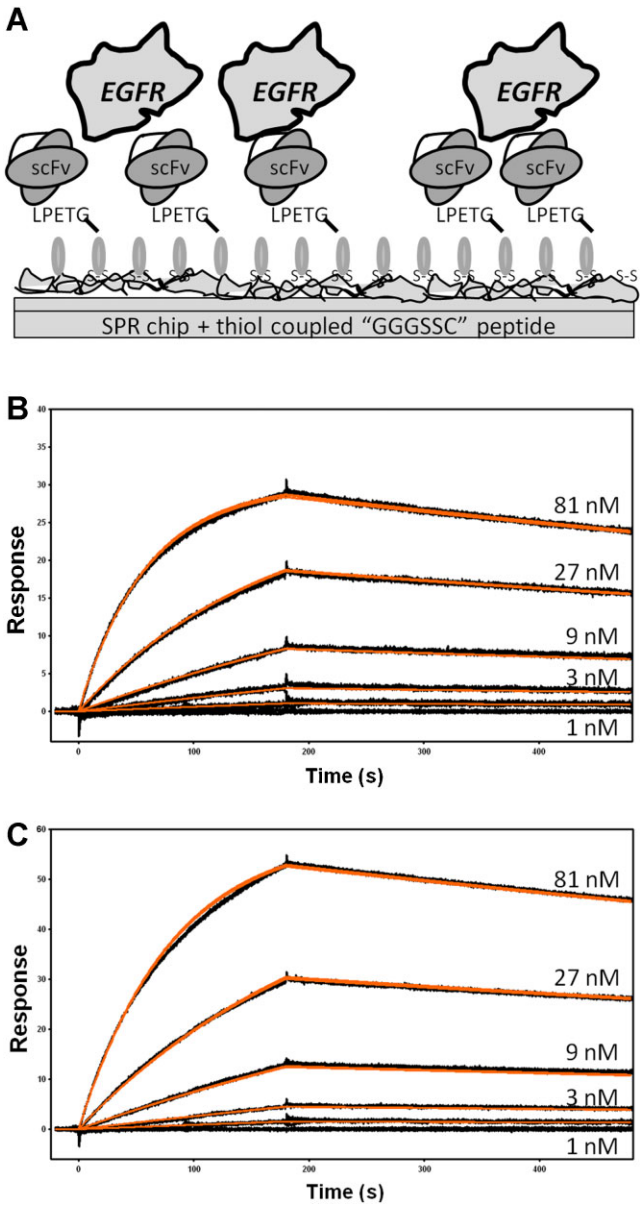

Figure 7. **A**: Cartoon illustrating in situ immobilization of sc528 to a CM5 biosensor chip through a thiol-conjugated poly-glycine linker and conjugation by SrtA ligation. The antibody fragments are uniformly oriented toward the EGFR analyte, allowing efficient kinetic analysis. **B**: Triplicate data sets for sEGFR501 (81, 27, 9, 3, 1 nM) binding sc528 coupled through sortase-mediated ligation. **C**: For comparison, triplicate data sets for sEGFR501 (81, 27, 9, 3, 1 nM) binding sc528 attached through direct amine coupling. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/bit]

cytokines resulted in full retention of biological activity in vitro and an enhanced serum half-life in vivo (Popp et al., 2011). In an extension of this work, the authors used the sortase transpeptidation reaction to generate cyclised forms of a number of cytokines, including erythropoietin, which were equipotent when compared to their linear counterparts while exhibiting enhanced thermostability. Similarly, efficient cyclization of a motogenic peptide, histatin, resulted in enhanced biopotency in a wound-healing model (Bolscher et al., 2011). An obvious application is the utilization of enzyme-mediated conjugation to accelerate the assembly and evaluation of antibody (and other protein) drug conjugates, particularly in oncology, although no examples have been provided to date. One of the critical stumbling blocks limiting the development of more therapeutically efficacious antibody-drug conjugates has been the challenge of achieving site-specific labeling of antibodies in a convenient, reproducible manner, yielding a controllable payload that is applicable to antibodies generally (Alley et al., 2010). Thus, it can readily be envisaged that sortase-mediated conjugation could be established as a platform technology for attaching, in a site-specific manner, a defined drug payload to an antibody/antibody fragment, or indeed any antigen-binding protein-based moiety, thereby accelerating the assembly/evaluation of protein-drug conjugates.

Our ability to combine the technologies of protein engineering of recombinant antibodies with sortase-mediated conjugation is illustrated by the coupling of sc528 to a biosensor surface. Like Clow et al. (2008), we utilized a thiol-coupled polyglycine peptide attached to a biosensor chip as the oligo-glycine acceptor molecule. Utilizing purified recombinant SrtA, we were able to reduce the coupling reaction time to 2 h in situ rather than the overnight reaction in a petri dish which was previously reported (Clow et al., 2008). Importantly, direct comparison with antigen coupled to a chip using the standard procedure of amine coupling

resulted in near identical affinities, indicating that sortase-mediated ligation is no worse, and may potentially be superior, to conventional methods. For example, although not demonstrated in the case of sc528, amine coupling typically results in heterogeneous ligand (antibody) immobilization that can compromises analyte binding capacity and overall estimated affinity (Kortt et al., 1997) due to amine coupling occurring via lysine residue(s) within or near the antibody paratope. Additionally, in situ sortase-mediated coupling allows for different ligands, such as scFv antibody mutants, to be immobilized in separate flow cells on the same chip. Similarly, taking advantage of the high specificity of this reaction we are currently investigating direct coupling to the sensor chip surface from crude protein sample without prior purification. If successful, this approach should allow development of systems for rapid immobilization of ligand surfaces toward rapid generation of high quality kinetic data sets.

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