Published in final edited form as:

Methods. 2014 January 1; 65(1): 133–138. doi:10.1016/j.ymeth.2013.05.023.

Antibody conjugation via one and two C-terminal selenocysteines

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Abstract

Conventional antibody conjugation methods generate antibody-drug conjugates that are heterogeneous mixtures with undefined stoichiometry and variable pharmacokinetic and pharmacodynamic properties. We have previously described a strategy to generate site-specific antibody conjugates by genetic engineering of an antibody with a single C-terminal selenocysteine, the 21st natural amino acid, which displays unique chemical reactivity allowing selective conjugation in the presence of all other natural amino acids. In the present work, we describe a method for expanding this technology to higher drug-to-antibody ratios by genetically engineering an antibody with two C-terminal selenocysteines. Both selenocysteines effectively conjugate to a fluorescent iodoacetamide derivative and the resulting conjugate fully retains its antigen binding capability. Our method provides a platform for creating stoichiometrically defined antibody-drug conjugates for therapeutic intervention.

Keywords

Antibody engineering; Antibody conjugation; Selenocysteine

1. Introduction

In the past 15 years, monoclonal antibody (mAb)-based cancer therapy has become an established therapeutic modality. However, due to efficacy limitations, most naked mAbs must be administered in combination with standard chemotherapies for the treatment of cancer, particularly for solid malignancies [1]. Antibody-drug conjugates (ADCs) that combine a mAb *carrier* and a cytotoxic small-molecule *payload* were developed to overcome the insufficient efficacy of naked mAbs while retaining their high binding specificity and affinity for tumor cell surface antigens and their long circulatory half-life.

Conventional antibody conjugation randomly utilizes cysteine (Cys) or lysine (Lys) residues to attach the drugs, resulting in the formation of a heterogeneous mixture of conjugates,

which likely have distinct pharmacokinetics, affinities, stabilities, efficacies, and safety profiles [2,3]. Site-specific antibody conjugation, on the other hand, permits homogeneous and stoichiometrically defined drug loading without impacting the efficacy of ADCs while further reducing their systemic toxicity [4]. The challenge of site-specific antibody conjugation is to introduce a unique chemical reactivity without altering the overall structure and function of the antibody molecule.

We previously reported a method for site-specific antibody conjugation by incorporation of a selenocysteine (Sec) at the C-terminus of antibodies in Fc, Fab, and IgG format [5–8]. Sec, the 21st natural amino acid, is analogous to Cys and serine (Ser), but has a selenium-containing selenol group in place of the sulfur-containing thiol group and the oxygen-containing hydroxyl group, respectively. The low pKa (5.2) of the nucleophilic selenol group in Sec endows this amino acid with unique biochemical properties, allowing regiospecific covalent conjugation with electrophilic compounds in the presence of the side chains of all other natural amino acids including the thiol group of Cys (pKa 8.3). Electrophilic compounds we have used for selective conjugation at the Sec interface include iodoacetamide and maleimide derivatives of biotin, fluorescein, and various cell surface receptor-targeting small molecules [5–9]. Sec is cotranslationally incorporated into proteins by recoding the UGA codon from termination to Sec insertion by mechanisms that are different in prokaryotes and eukaryotes and depend on structural features of the mRNA. Sec insertion in eukaryotes requires the presence of the Sec insertion machinery and a Sec insertion sequence (SECIS) element, a specific mRNA secondary structure, which is located in the 3'-untranslated region (3'-UTR) [10]. Components of the eukaryotic Sec insertion machinery include enzymes catalyzing the conversion of Ser-tRNA^{Sec} to Sec tRNA^{Sec} and proteins directly assisting in Sec insertion by binding to Sec-tRNA^{Sec} or SECIS [11]. All of these components are naturally available in mammalian cell lines that are commonly used for ectopic antibody expression at laboratory to manufacturing scale.

We developed a method for mammalian cell expression of the human IgG1-derived Fc protein with a C-terminal Sec residue by introducing an engineered SECIS element from the 3'-UTR of the cDNA of human selenoprotein thioredoxin reductase 1 (TXNRD1), an enzyme with a natural C-terminal Sec residue, into the expression cassette (Fig. 1A) [5]. This genetically engineered Fc protein displays a unique chemical reactivity that affords for selective conjugation to a variable small synthetic molecule at the Sec interface. Based on this platform, we generated recombinant whole antibody molecules (IgG) and antibody fragments (Fab) with a C-terminal Sec residue and demonstrated that their Sec conjugates fully retained their antigen binding capability and, in the case of IgG, the ability to mediate effector functions [6].

Incorporation of one Sec residue at the C-terminus will enable one antibody to carry only one drug, i.e., afford a drug-to-antibody ratio (DAR) of 1, which limits the efficacy of the ADCs. To address this issue, we here describe a method for generating an antibody with two C-terminal Sec residues in a single chain Fv-Fc (scFv-Fc) format, and demonstrate that both Sec can efficiently conjugate to a fluorescent iodoacetamide derivative, providing a rationale for applying our Sec interface technology to the generation of ADCs with a stoichiometrically defined DAR of 2.

2. Materials and methods

2.1. Sec protein engineering

2.1.1. R11-scFv-Fc-Sec-His—To express R11-scFv-Fc with one C-terminal Sec residue, we first generated mammalian cell expression vector pCEP4-Fc-Sec-His as described previously [5]. This construct contains the hinge-C_H2–C_H3 sequence of human IgG1,

featuring a Cys-to-Ser mutation in the hinge and a Lys-to-Ala mutation at the C-terminus, followed by a TGA codon, six His codons, a TAA codon, and a SECIS element from the 3'-UTR of the cDNA of human TXNRD1. The chimeric rabbit/human anti-human ROR1 mAb R11 in IgG format [12] was converted to scFv-Fc format following our established protocol [13]. DNA encoding the variable domains of R11 linked with a (Gly₄Ser)₃ linker was optimized for expression in human cells by custom synthesis (DNA2.0) and cloned into pCEP4-Fc-Sec-His by *KpnI/SapI* ligation. The resulting plasmid was designated pCEP4-R11-scFv-Fc-Sec-His.

2.1.2. R11-scFv-Fc-Sec-Sec-His—To express R11-scFv-Fc with two C-terminal Sec residues, DNA with two TGA codons and two identical SECIS elements from human TXNRD1 were custom synthesized as gBlocks Gene Fragments (Integrated DNA Technologies). The two TGA codons were spaced by a sequence encoding a Gly₄Ser linker. The second SECIS element was located 158 bp downstream from the first. This DNA fragment was amplified by PCR and cloned into pCEP4-R11-scFv-Fc-Sec-His by *HindIII/XhoI* ligation. The resulting plasmid was designated pCEP4-R11-scFv-Fc-Sec-Sec-His. All constructs were verified by DNA sequencing.

2.2. Sec protein expression and purification

The mammalian cell expression vectors described above were transiently transfected into Human Embryonic Kidney (HEK) 293F cells (Life Technologies) with 293Fectin Transfection Reagent (Life Technologies) using condition detailed in the manufacturer's protocol. Transfected HEK 293F cells were cultured in protein-free FreeStyle 293 Expression Medium (Life Technologies), supplemented with 1 μ M sodium selenite (Na₂SeO₃; Sigma-Aldrich), in spinner flasks (Integra Biosciences) under constant rotation at 75 rpm in a humidified atmosphere containing 8% CO₂ at 37 °C. Three days after transfection, the medium was collected after centrifugation, replaced for two additional days, and collected again. This procedure was repeated once for an additional two days. The combined supernatants were filtered through a 0.45- μ m membrane and 10-fold concentrated using an ultrafiltration device with a 30-kDa cutoff membrane (Millipore). The concentrate was diluted 1:1 with phosphate-buffered saline (pH 7.4; PBS) and loaded on a 1-mL recombinant Protein G column (HiTrap; GE Healthcare) connected to an ÄKTApurifier system (GE Healthcare). PBS was used for column equilibration and washing, 0.5 M acetic acid (pH 3.0) for elution, and 1 M Tris-HCl (pH 8.0) for immediate neutralization. The neutralized eluate was buffer-exchanged with PBS and concentrated simultaneously using 30-kDa cutoff centrifugal filter devices (Millipore). In order to separate scFv-Fc-Sec-His (or scFv-Fc-Sec-Sec-His) protein from scFv-Fc-stop protein, the purified scFv-Fc protein mixture was 10-fold diluted in loading/washing buffer (500 mM NaCl and 25 mM imidazole in PBS) and loaded on a 1-mL immobilized metal ion affinity chromatography (IMAC) column (HisTrap, GE Healthcare) connected to the ÄKTApurifier system. The flow-through of the column containing the scFv-Fc-stop protein was collected. Subsequently, the column was washed with 50 mL loading/washing buffer and the bound scFv-Fc-Sec-His (or scFv-Fc-Sec-Sec-His) protein was eluted with elution buffer (500 mM NaCl and 500 mM imidazole in PBS). Both eluate and flow-through were buffer-exchanged with PBS and concentrated as described above.

2.3. Selective conjugation

For selective conjugation at the Sec interface, R11-scFv-Fc-Sec-His, R11-scFv-Fc-Sec-Sec-His, and the negative control R11-scFv-Fc-stop proteins were diluted in 15 mL 100 mM sodium acetate (pH 5.2) and concentrated to 4 μ M (~0.5 mg/mL) using a 30-kDa cutoff centrifugal filter device. The proteins were reduced by incubation with 4 mM dithiothreitol (DTT) for 20 min at room temperature, followed by incubation with 5-

iodoacetamidofluorescein (5-IAF; Thermo Scientific) at a final concentration of 80 μ M for 0.5–1 h at room temperature in the dark. Reactions were quenched by addition of 2% (v/v) β -mercaptoethanol. Following conjugation to 5-IAF, the proteins were diluted in 15 mL 100 mM sodium acetate (pH 5.2) and concentrated to 250 μ L as described above. This step was repeated once with 15 mL 100 mM sodium acetate (pH 5.2) and subsequently twice with 15 mL PBS to remove unconjugated compounds. Conjugates in PBS were stored refrigerated (4 $^{\circ}$ C) for short term use and frozen (-80° C) in aliquots for long term use.

2.4. Electrophoresis

2.4.1. Western blotting—Supernatants from transiently transfected HEK293F cells were incubated at 70 $^{\circ}$ C for 5 min in NuPAGE LDS Sample Buffer (Life Technologies) supplemented with 2% (v/v) β -mercaptoethanol (MP Biomedicals, LLC) and electrophoresed on a NuPAGE Novex Bis–Tris 4–12% gradient gel (Life Technologies), blotted on a PVDF membrane (Millipore), blocked with 5% (w/v) non-fat milk, and incubated with mouse mAb Penta-His (Qiagen) diluted to 0.5 μ g/mL in 3% BSA/PBS followed by HRP-coupled goat anti-mouse IgG polyclonal antibodies (pAbs; Jackson ImmunoResearch Laboratories) diluted 1:10,000 in 5% (w/v) non-fat milk. HRP-coupled donkey anti-human IgG Fc γ pAbs (Jackson ImmunoResearch Laboratories) were used as positive control. Immunoreactive bands were developed using ECL Prime Western Blotting Detection Reagent (GE Healthcare) and visualized using Blue Basic autoradiography film (GeneMate).

2.4.2. SDS-PAGE—A 10- μ L aliquot of samples from each of the stopped conjugation reaction solutions was incubated at 70 $^{\circ}$ C for 5 min in NuPAGE LDS Sample Buffer supplemented with 2% (v/v) β -mercaptoethanol and then loaded on a NuPAGE Novex Bis–Tris 4–12% gradient gel. Following SDS–PAGE and prior to staining with SimplyBlue SafeStain (Life Technologies), a picture of the gel was taken under blue light illumination (Life Technologies) to record the fluorescence. Quantification of band intensity was computed with NIH ImageJ software.

2.5. ELISA

For coating, each well of a 96-well Costar 3690 plate (Corning) was incubated with 100 ng recombinant human ROR1 extracellular domain (hROR1ECD) [12] in 25 μ L PBS for 1 h at 37 $^{\circ}$ C. After blocking with 150 μ L 3% (w/v) BSA/PBS for 1 h at 37 $^{\circ}$ C, the plate was incubated with R11-scFv-Fc-stop, R11-scFv-Fc-Sec-His or R11-scFv-Fc-Sec-Sec-His protein at 0.4 μ g/mL (10 ng/well) for 2 h at 37 $^{\circ}$ C. After washing with water (10 $\times$ 200 μ L/well), the plate was incubated with 50 μ L of either mouse mAb Penta-His (1:500 dilution) or HRP-coupled donkey anti-human IgG Fc γ pAbs (1:1,000 dilution) in 1% (w/v) BSA/PBS for 1 h at 37 $^{\circ}$ C. The plate was washed with water as before, and colorimetric detection was performed using 2, 2'-azino-bis (3-ethylbenzthiazoline)-6-sulfonic acid (ABTS; Roche) as substrate according to the manufacturer's directions. For His-tag detection, before adding the substrate, the plate was incubated with HRP-coupled goat anti-mouse IgG pAbs (1:1000 dilution) in 1% (w/v) BSA/PBS for 1 h at 37 $^{\circ}$ C followed by washing with water as before. The ELISA with 5-IAF-labeled and unlabeled R11-scFv-Fc proteins over a concentration range of 0.03–300 nM was carried out as above, using HRP-coupled donkey anti-human IgG Fc γ pAbs for detection.

2.6. Flow cytometry

Human mantle cell lymphoma HBL-2 and Burkitt Lymphoma Raji cells (American Type Culture Collection) maintained in 10% (v/v) FBS in RPMI 1640 medium supplemented with penicillin-streptomycin (all Life Technologies) were collected by centrifugation,

resuspended in ice-cold 0.5% (w/v) BSA in PBS (flow cytometry buffer), and aliquots of 50 μL containing 5×10^5 cells were distributed into a V-bottom 96-well plate (Corning). The cells were first blocked with 10 μg polyclonal human IgG (Thermo Scientific) for 30 min on ice and then incubated with 3 μg 5-IAF-labeled R11-scFv-Fc-Sec-His, R11-scFv-Fc-Sec-Sec-His, or R11-scFv-Fc-stop proteins for 30 min on ice. After washing twice with ice-cold flow cytometry buffer, the cells were resuspended in 400 μL flow cytometry buffer and analyzed using an LSR II Flow Cytometer (Becton-Dickinson).

3. Results and discussion

3.1. Cloning, expression, and purification of Sec and Sec-Sec protein

We previously reported a mammalian cell expression system for the generation of a genetically engineered Fc protein with a C-terminal Sec residue followed by a His tag (Fc-Sec-His) [5]. The corresponding mammalian cell expression vector consisted of an exon/intron gene sequence encoding the human IgG1-derived Fc fragment (hinge- $\text{C}_{\text{H}}2$ - $\text{C}_{\text{H}}3$) fused to a TGA codon, followed by a His₆-encoding sequence, a TAA stop codon, and a SECIS element from the 3'-UTR of the cDNA of human TXNRD1 (Fig. 1A). R11 is a chimeric rabbit/human anti-human ROR1 mAb that was generated by phage display technology [12]. To convert the Fc-Sec-His cassette to a R11-scFv-Fc-Sec-His cassette (Fig. 1B), we reformatted R11 from IgG1 to scFv-Fc and cloned it into the Fc-Sec-His vector using an internal *SapI* restriction site located in the $\text{C}_{\text{H}}2$ domain [13]. R11-scFv-Fc-Sec-Sec-His was generated by replacing the Sec-His encoding sequence in the R11-scFv-Fc-Sec-His cassette with a Sec-Gly₄Ser-Sec-His encoding sequence (Fig. 1C). In this construct, we duplicated the SECIS element from TXNRD1 based on the observation that the 3'-UTR of methionine sulfoxide reductase from *Metridium senile* contains two nearly identical SECIS elements, which collaborate for the insertion of four Sec residues at the C-terminus of the protein [14]. The 3'-UTR of human selenoprotein P, which harbors nine Sec residues among its C-terminal 82 amino acids, also contains two SECIS elements, suggesting that more than one SECIS element may be necessary for efficient incorporation of multiple Sec residues [15].

Both R11 constructs with either one or two TGA codons were transiently transfected into HEK 293F cells that were maintained in protein-free medium supplemented with 1 μM sodium selenite. We had previously found that adding a selenium source to the medium increases the yield of Fc-Sec-His protein by factor 10 [5]. Analogous to Fc-Sec-His, R11-based scFv-Fc-Sec-His and scFv-Fc-Sec-Sec-His proteins were purified from supernatants by a tandem column chromatography process [5]. Protein G affinity chromatography was used to purify the total scFv-Fc protein, followed by IMAC to separate scFv-Fc-Sec-His and scFv-Fc-Sec-Sec-His (the product of Sec insertion at UGA) from scFv-Fc-stop (the product of termination at UGA). Importantly, scFv-Fc-stop protein is an inevitable byproduct of our mammalian cell expression system as termination at the UGA codon typically dominates Sec insertion and read-through, despite the presence of a SECIS element [10]. The yields of total protein for both constructs were similar to what we previously reported for Fc (10 mg/L) [5]. The ratio of scFv-Fc-Sec-His to scFv-Fc-stop and the ratio of scFv-Fc-Sec-Sec-His to scFv-Fc-stop were also comparable to the ratio of Fc-Sec-His to Fc-stop (~1:4) [5]. With the observed ~1:4 ratio of Sec insertion to termination at the UGA codon, the proportion of the scFv-Fc-Sec-His homodimer containing two Sec residues makes up only 1% of total protein, assuming a binomial distribution ($p + q = 1$) with free pairing of the Fc fragments ($p^2 + 2pq + q^2 = 1$). In case of the scFv-Fc-Sec-Sec-His construct, it is theoretically possible that the translation stop at the second UGA codon results in a product of scFv-Fc-Sec-stop protein, which could form an scFv-Fc-Sec-stop/scFv-Fc-Sec-Sec-His heterodimer containing three Sec residues. However, scFv-Fc-Sec-stop does not exist at detectable concentrations

according to our conjugation studies described below. Therefore, similar to scFv-Fc-Sec-His, the major fraction of scFv-Fc-Sec-Sec-His protein after IMAC purification is the scFv-Fc-Sec-Sec-His/scFv-Fc-stop heterodimer. The scFv-Fc-Sec-Sec-His/scFv-Fc-Sec-Sec-His homodimer containing four Sec residues is predicted to make up only 1% of total protein.

We also engineered an R11-scFv-Fc protein with three Sec residues at the C-terminus. The expression level of this protein significantly dropped compared to that of R11-scFv-Fc with one or two Sec residues, which were expressed and purified with similar yields (Fig. 2A), suggesting that the efficient incorporation of more than two C-terminal Sec residues is a challenge to the Sec insertion machinery in our current mammalian cell expression system. Identifying and supplying limiting components of the eukaryotic Sec insertion machinery [11] may enable an increase of the ratio of Sec insertion to termination at the UGA codon and lead to a more efficient incorporation of three or more Sec residues in one polypeptide.

To determine if the incorporation of one and two Sec residues at the C-terminus of R11-based scFv-Fc influences antigen binding, we compared the binding of R11-scFv-Fc-stop, R11-scFv-Fc-Sec-His, and R11-scFv-Fc-Sec-Sec-His to the immobilized recombinant human ROR1 extracellular domain (hROR1ECD) by ELISA. As shown in Fig. 2B, no difference in antigen binding was detected. Thus, these newly engineered Sec and Sec-Sec proteins were used for further site-specific conjugation studies at the Sec interface.

3.2. Selective conjugation at both Sec residues

We previously demonstrated that engineered Fc-Sec, IgG-Sec, and Fab-Sec display unique chemical reactivity, allowing selective conjugation at the Sec interface under mildly acidic and reducing conditions [5–8]. To investigate whether both Sec residues in the newly engineered Sec-Sec protein could be selectively conjugated, we labeled R11-scFv-Fc-Sec-Sec-His, R11-scFv-Fc-Sec-His, and R11-scFv-Fc-stop with a commercially available iodoacetamide derivative of fluorescein (5-IAF) under the same acidic but stronger reducing conditions as what we previously established [5]. As shown in Fig. 3, we anticipated that the two Sec residues form a diselenide bridge (Se–Se) based on the observation that a diselenide bond was formed between two Sec residues in SelL, a family of prokaryotic and eukaryotic selenoproteins, containing two Sec residues separated by two other amino acid residues [16]. Due to the lower redox potential of the Se–Se bond (–381 mV) compared to the S–S bond in DTT (–323 mV), a large molar excess (>300-fold) of DTT was needed to fully reduce the diselenide bridge [17]. Here we show that in the presence of a 1,000-fold molar excess of DTT, R11-scFv-Fc-Sec-Sec-His was successfully labeled with fluorescence. Under the same conditions, R11-scFv-Fc-Sec-His was also labeled with fluorescence but to a lesser extent (~50%) than R11-scFv-Fc-Sec-Sec-His, whereas R11-scFv-Fc-stop remained unlabeled (Fig. 4A). These results indicate that selective conjugation occurred at both Sec residues. Notably, the scFv-Fc-stop protein separated from the scFv-Fc-Sec-Sec-His protein by IMAC was not labeled under the same conditions (data not shown), suggesting that termination occurs dominantly at the first but not second UGA codon and that scFv-Fc-Sec-Sec-His constitutes a dimeric protein with a single Sec-Sec displaying C-terminus.

To show that the resulting conjugates retain their antigen binding capability, ELISA and flow cytometry analyses were carried out. As shown in Fig. 4B, 5-IAF-labeled and unlabeled R11-scFv-Fc proteins bound equally to immobilized hROR1ECD with an EC₅₀ of 5 nM. Flow cytometry analysis revealed that R11-scFv-Fc proteins labeled through one and two C-terminal Sec residues recognized ROR1-positive HBL-2 cells but not ROR1-negative Raji cells, indicating that the binding is ROR1-specific (Fig. 5). A larger shift was observed for the binding of HBL-2 cells by the R11-scFv-Fc-Sec-Sec-His/5-IAF conjugate compared to the R11-scFv-Fc-Sec-His/5-IAF conjugate, further demonstrating the increased loading capacity afforded by two C-terminal Sec residues (Fig. 5).

4. Conclusions

Traditional antibody conjugation methods using Lys or Cys residues can generate mixtures of above 10^6 species [3], challenging manufacturing and regulatory approval in the development of antibody-drug conjugates. We previously developed a Sec interface technology to generate homogeneous antibody conjugates. Here we further improved this method by incorporation of two C-terminal Sec residues for a stoichiometrically defined DAR of 2. Site-specific conjugation with a DAR of 2 was previously shown to afford ADCs with similar efficacy to randomly labeled ADCs but with an improved therapeutic index in *in vivo* models [4]. Thus, the Sec interface technology provides an applicable platform for creating next-generation ADCs for cancer therapy. Arming scFv-Fc-Sec-Sec-His proteins, which target ROR1 and other tumor cell surface antigens, with cytotoxic drugs is ongoing in our laboratory.

Acknowledgments

This is manuscript 24013 from The Scripps Research Institute.

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Fig. 1.

Fc-Sec, scFv-Fc-Sec, and scFv-Fc-Sec-Sec engineering. (A) Schematic of the Fc-Sec expression cassette, which encodes the two C-terminal constant domains (C_{H2} and C_{H3} ; gray) preceded by the hinge region (H; black) of the heavy chain of human IgG1. A C-terminal Sec residue (red) followed by a His₆ tag (black) was introduced by combining a TGA codon (red) with a 3'-UTR that contains a SECIS element. After IMAC purification, the dominant product of this expression cassette is a dimeric Fc protein with a single Sec-His₆-displaying C-terminus. (B) Schematic of the scFv-Fc-Sec expression cassette for the expression of a dimeric scFv-Fc protein with a single Sec-His₆-displaying C-terminus. V_L and V_H denote variable light (white) and heavy chain (gray) domains spaced by a peptide linker (L; black). (C) Schematic of the scFv-Fc-Sec-Sec expression cassette for the expression of a dimeric scFv-Fc protein with a single Sec-Gly₄Ser-Sec-His₆-displaying C-terminus. Note the two SECIS elements in the 3'-UTR.

Fig. 2.

Expression of chimeric rabbit/human anti-human ROR1 mAb R11 in scFv-Fc format with multiple C-terminal Sec residues. (A) Supernatants from HEK293F cells transiently transfected with mammalian cell expression vectors encoding R11-scFv-Fc without C-terminal Sec residue (lane 0), with one Sec residue (lane 1), with two Sec residues (lane 2), and with three Sec residues (lane 3) were subjected to reducing SDS-PAGE followed by Western blotting using a mouse anti-His mAb and HRP-coupled goat anti-mouse IgG pAbs for detection (anti-His; *top*). HRP-coupled donkey anti-human IgG Fc γ pAbs were used as loading control (anti-Fc; *bottom*). (B) Binding of R11-scFv-Fc protein with or without C-terminal Sec residues to immobilized hROR1ECD was detected with either anti-His (*gray*) or anti-Fc (*black*) as described above. Shown is the mean $\pm$ standard deviation of triplicates. (1) PBS alone; (2) R11-scFv-Fc-stop; (3) R11-scFv-Fc-Sec-His; (4) R11-scFv-Fc-Sec-Sec-His.

Fig. 3. Schematic for the conjugation of an iodoacetamide derivative to an antibody in scFv-Fc format with two C-terminal Sec residues. First, 4 μ M scFv-Fc-Sec-Sec-His protein is reduced in a 20-min pre-incubation with 4 mM DTT in 100 mM NaOAc (pH 5.2). This is followed by a 30-min incubation with 80 μ M of an iodoacetamide derivative of a, e.g., fluorescent or cytotoxic compound in the presence of 4 mM DTT in the same buffer.

Fig. 4.

Characterization of selective conjugation of R11-based scFv-Fc to 5-IAF at the Sec interface. (A) Reducing SDS-PAGE analysis of R11-scFv-Fc-stop (lane 2), R11-scFv-Fc-Sec-His (lane 3), and R11-scFv-Fc-Sec-Sec-His (lane 4) (4 μ g) conjugated to 5-IAF under the conditions described in Fig. 3. R11-scFv-Fc-stop without any incubation (lane 1) was used as negative control, and a protein marker (in kDa) was run in lane 0. Fluorescence was first recorded with blue light (*top*), followed by Coomassie staining of the same gel (*bottom*). The indicated band intensity ratio was computed with NIH ImageJ software and normalized with lane 3 set at 1. (B) Following selective conjugation to 5-IAF, the binding of R11-scFv-Fc proteins over a concentration range of 0.03–300 nM to immobilized hROR1ECD was detected with HRP-coupled donkey anti-human IgG Fc γ pAbs. Shown is the mean $\pm$ standard deviation of triplicates.

Fig. 5. Cell surface binding. Flow cytometry analysis of the binding of 5-IAF-labeled R11-scFv-Fc-Sec-His (*blue* line) and R11-scFv-Fc-Sec-Sec-His (*red* line) to ROR1-positive HBL-2 cells and ROR1-negative Raji cells. R11-scFv-Fc-stop incubated with 5-IAF (*green* line) did not reveal any staining above background (*black* line) on either cell line.