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TITLE:

Preparation of Site-Specific Cytotoxic Protein Conjugates via Maleimide-thiol Chemistry and Sortase A-Mediated ligation

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SUMMARY:

Here we provide detailed protocols for a site-specific labeling of proteins with cytotoxic drugs using maleimide-thiol reaction and sortase A-mediated ligation.

ABSTRACT:

Cancer is currently the second most common cause of death worldwide. The hallmark of cancer cells is the presence of specific marker proteins such as growth factor receptors on their surface. This feature enables development of highly selective therapeutics, the protein bioconjugates, composed of targeting proteins (antibodies or receptor ligands) connected to highly cytotoxic drugs by a specific linker. Due to very high affinity and selectivity of targeting proteins the bioconjugates recognize marker proteins on the cancer cells surface and utilize receptor-mediated endocytosis to reach the cell interior. Intracellular vesicular transport system

ultimately delivers the bioconjugates to the lysosomes, where proteolysis separates free cytotoxic drugs from the proteinaceous core of the bioconjugates, triggering drug-dependent cancer cell death. Currently, there are several protein bioconjugates approved for cancer treatment and large number is under development or clinical trials.

One of the main challenges in the generation of the bioconjugates is a site-specific attachment of the cytotoxic drug to the targeting protein. Recent years have brought a tremendous progress in the development of chemical and enzymatic strategies for protein modification with cytotoxic drugs. Here we present the detailed protocols for the site-specific incorporation of cytotoxic warheads into targeting proteins using a chemical method employing maleimide-thiol chemistry and an enzymatic approach that relies on sortase A-mediated ligation. We use engineered variant of fibroblast growth factor 2 and fragment crystallizable region of human immunoglobulin G as an exemplary targeting proteins and monomethyl auristatin E and methotrexate as model cytotoxic drugs. All the described strategies allow for highly efficient generation of biologically active cytotoxic conjugates of defined molecular architecture with potential for selective treatment of diverse cancers.

INTRODUCTION:

Decades of scientific efforts have led to an enormous advancement in our knowledge about the molecular mechanisms governing cancer development and progression. At the same time, the therapeutic possibilities are still largely limited due to the adverse effects of drugs caused by their lack of selectivity, the great variability of tumors and drug-resistance developed after prolonged treatment. Targeted anti-cancer therapies have been gaining attention in recent years as novel and highly promising approaches for treatment of diverse tumors. Targeted therapies rely on sophisticated drug delivery systems that precisely deliver the cytotoxic payload to the cancer cells and spare the healthy ones. These include mainly diverse nanoparticles, liposomes, and protein-based drug carriers.

Cancer cells often expose elevated levels of specific marker proteins on their surface. Antibody drug conjugates (ADCs) are novel protein-based anti-cancer therapeutics, which combine in one molecule extreme specificity of monoclonal antibodies and high cytotoxic potency of drugs. Once bound to the cancer cell surface, ADC utilize receptor-mediated endocytosis to enter the cell. Subsequently, ADCs are transported via endosomal compartments to the lysosomes, where proteases degrade ADCs and release active cytotoxic drugs. Currently, there are eight ADCs approved in the US for the treatment of diverse tumors, including triple negative breast cancer, HER2 positive breast cancer, urothelial cancer, diffuse large B-cell lymphoma, hematological malignancy, Hodgkin lymphoma and acute myeloid leukemia. Large number of ADCs are also either under development or await approval[1]. Noteworthy, protein engineering approaches have led to the development of diverse alternative to monoclonal antibodies protein scaffolds and their cytotoxic conjugates. These include different antibody fragments[2,3], DARPins[4,5], knottins[6,7], centyrins[8], affibodies[9,10], or engineered receptor ligands[11,12].

There are several critical requirements that have to be met by a successful protein-based cytotoxic conjugate, namely the conjugate stability, extraordinary specificity, high affinity of the

conjugate towards cancer-specific marker, rapid internalization of the conjugate into the cancer cell interior, its efficient transport to the lysosomes and effective intracellular release of the active payload. Another important feature is conjugates homogeneity, which largely depends on the applied strategy for the attachment of the payload to the targeting proteins. There are several methods available for site-specific conjugation of proteins with cytotoxic drugs, like modification of protein side-chain cysteine or lysine residues, attachment of the drug to unnatural amino acids incorporated into the targeting proteins, or enzymatic modifications of the targeting proteins (e.g., with transglutaminase, glycosyltransferase, formylglycine-generating enzyme, sortase A). In most cases site-specific conjugation methods require modifications of the targeting molecules (e.g., via cysteine engineering or introduction of short peptide tags), but in turn result in an efficient production of homogenous conjugate of interest.

Here we provide protocols for highly efficient site-specific conjugation of targeting proteins with cytotoxic drugs. As exemplary proteins we used two different molecules: the fragment crystallizable (Fc) of human IgG and an engineered variant of human fibroblast growth factor 2 (FGF2). The Fc fragment constitutes integral part of typical ADCs, but it is also present in other types of conjugates like cytotoxic peptibodies or conjugates of antibody fragments. FGF2 is a natural fibroblast growth factor receptor (FGFR) ligand that was successfully engineered to yield a selective cytotoxic conjugate targeting FGFR-overproducing cancer cells.

We present two distinct conjugation strategies allowing for the site-specific incorporation of cytotoxic drugs. First, the protocol for conjugation to the cysteine side chains of the Fc fragment via maleimide-thiol chemistry based on Hermanson's protocol[13] is provided (**Figure 1A,B**). In this protocol two disulfide linkages are initially reduced with tris(2-carboxyethyl)phosphine (TCEP) and resulting free thiol groups are subjected to conjugation with monomethyl auristatin E (MMAE) via maleimide-thiol chemistry (**Figure 1B**). Due to the interaction between constant heavy chain domains 2 and 3 (CH2 and CH3) the dimeric structure of the drug-linked Fc is preserved. Secondly, the strategy for the generation of double warhead FGF2 conjugate is presented that combines cysteine engineering and sortase A-mediated ligation for incorporation of two distinct drugs into FGF2 in a site-specific manner (**Figure 1A,C**). The cysteine-free variant of FGF2 bearing additional N-terminal KCKSGG with single exposed cysteine residue and C-terminal LPETGG short peptide tag is used[12]. The maleimide-thiol reaction allows for the conjugation of MMAE to cysteine within KCKSSG linker designed by our group[14,15]. Sortase A-dependent step (based on Chen et al.)[16] mediates the ligation of methotrexate (MTX)-linked tetraglycine peptide GGGG-MTX to the C-terminal LPETGG sequence, yielding two types of single warhead conjugates (**Figure 1C**). Sortase A is a cysteine protease that catalyzes the transpeptidation reaction between LPETGG and GGGG motifs. The enzyme binds to the LPETGG motif at the C-terminus of the protein, then the amide bond between the threonine and the glycine is hydrolyzed to form an enzyme-substrate complex. The next step is the aminolysis of the thioester enzyme-substrate bond, where the donor of a primary amino group is the glycine residue of the tetraglycine motif[17]. Combination of these two approaches generates site-specific FGF2 double warhead conjugates (**Figure 1C**). In principle, provided conjugation protocols can be successfully applied to any engineered targeting protein of interest to generate selective cytotoxic conjugates. Moreover, versatility of this approach makes it suitable for many other

protein-protein and protein-peptide ligation purposes, as well as for the attachment of lipids, polymers, nucleic acids and fluorophores to proteins with available sulfhydryl group (or generated by reduction of native disulfide bonds) and/or with introduced small peptide tag.

PROTOCOL:

1. Conjugation of the Fc-domain with MMAE

NOTE: Prior to site-specific conjugations prepare key reagents: highly pure protein of interest (in this case the Fc fragment and engineered FGF2 variant, as a starting point 1-5 mg of recombinant protein, prepared according to Sokolowska-Wedzina[18]), maleimidocaproyl-Val-Cit-*p*-aminobenzyl alcohol (PABC)-monomethyl auristatin E (MMAE) (CAUTION, highly cytotoxic agent, handle with care), Tris(2-carboxyethyl)phosphine (TCEP) and sortase A[16]. Methotrexate linked to tetraglycine peptide (GGGG-MTX) (CAUTION, MTX is a highly cytotoxic agent, handle with care) can be either synthesized on the solid support phase according to the solid phase peptide synthesis (SPPS) method in the Fmoc strategy[19] or obtained from commercial sources.

1.1. To reduce disulfide bonds within the Fc fragment (**Figure 1**), add tenfold molar excess of TCEP over the Fc protein (39 µM) in PBS (100 mM NaCl, 18 mM NaH_2PO_4, 33 mM Na_2HPO_4 pH 7.4) and incubate at 37 ˚C for 1 h. TCEP stock solution should have pH adjusted with 0.1 M NaOH to 6.0, in order to prevent protein precipitation from pH changes.

1.2. After incubation, filter the reduced protein using 0.2 µm filter to remove protein precipitates.

1.3. To the new reaction tube add first four-fold molar excess of MMAE (dissolve MMAE in *N,N*-dimethylacetamide (DMAc) to prepare 40 mM stock solution) over protein -SH groups. Then add double volume of PBS buffer in relation to the volume of the Fc protein. At last add reduced Fc fragment from step 1.2.

NOTE: In this step, order of reagents addition is important.

1.4. Carry out the conjugation reaction overnight at 15 ˚C with gentle rotation. These mild conditions prevent protein denaturation.

1.5. Filter the solution with conjugate using 0.2 µm filter to remove potential precipitates.

1.6. Carry out SDS-PAGE to analyze the efficiency of the reaction. Use 10% separation gel, 10 – 250 kDa protein marker, and analyze bands between 35 and 50 kDa (**Figure 2**).

NOTE: The protocol can be paused here, and the reaction mixture stored either at 4 ˚C for short term storage, or at -80 ˚C for long term storage. However, freezing and thawing can cause additional precipitation and decrease conjugate purification yields.

1.7. Load the reaction mixture on a column with protein A-conjugated beads and wash off the excess of MMAE with Washing buffer 1 containing 300 mM NaCl, 18 mM NaH_2PO_4, 33 mM, Na_2HPO_4, 0.1% Tween 20, 2 mM EDTA pH 7.4. Then wash the column using Washing Buffer 2 with 650 mM NaCl, 18 mM NaH_2PO_4, 33 mM Na_2HPO_4, pH 7.4.

NOTE: Through selective interaction of Fc region of the conjugate with protein A on the beads, the conjugate is captured on the column, while unreacted MMAE is washed out. This step is necessary to obtain highly pure conjugate.

1.8. Elute the Fc-MMAE conjugate from the column with 0.1 M sodium citrate pH 3.5 to 1.5 mL tubes containing 1 M Tris pH 9.0 (1 mL of protein to 200 µL of 1 M Tris pH 9.0).

1.9. Desalt Fc-MMAE to PBS pH 7.4 using a column with Sephadex G-25 resin. This step prevents denaturation of the conjugate and allows the conjugate to be used in in vitro and in vivo tests.

NOTE: The protocol can be paused here.

1.10. Carry out SDS-PAGE (10%) to analyze the final Fc-MMAE conjugate. Use 10% separation gel, 10 – 250 kDa protein marker, and analyze bands between 35 and 50 kDa (**Figure 2**).

2. Conjugation of engineered FGF2

2.1. Conjugation of GGGG-MTX to the C-terminal LPETGG sequence of engineered FGF2 via sortase A-mediated ligation

2.1.1. Transfer purified engineered FGF2 containing N-terminal KCKSGG and C-terminal LPETGG sequence (used in this step for conjugation) to the sortase A Reaction Buffer (25 mM HEPES pH 7.4, 150 mM NaCl, 10 mM $(NH_4)_2SO_4$, 5 mM $CaCl_2$) using a column with G-25 resin. Adjust the protein concentration to 1 µM with sortase A Reaction Buffer.

2.1.2. Add GGGG-MTX peptide dissolved in DMAc, directly to the protein solution to a final concentration of 100 µM.

2.1.3. Add sortase A to a final concentration of 0.1 µM and incubate for 12 h at 15 °C with gentle rotation. The concentrations of reagents to be used in steps 2.1.1-2.1.3 are largely determined by the yield of conjugate to be achieved and the biochemical characteristics of the targeting protein (protein purification effectiveness, its solubility and stability). For effective sortase A reaction, use a starting point ten- to hundred-fold times lower concentration of sortase A than the LPETGG-containing target protein and 100 times higher concentration of GGGG-MTX than LPETGG-tagged protein.

2.1.4. Load the reaction mixture on a Heparin Sepharose column.

2.1.5. Wash off unreacted molecules with 25 mM HEPES pH 7.4.

2.1.6. Elute the product of the reaction (FGF2.MTX) with 25 mM HEPES pH 7.4 with 2 M NaCl.

NOTE: The protocol can be paused here.

2.1.7. Analyze the efficiency of the conjugation using SDS-PAGE. Use 15% separation gel, 10 – 250 kDa protein marker, and analyze bands between 15 and 25 kDa (**Figure 3**).

2.2. Conjugation of MMAE to the N-terminal KCKSGG sequence of engineered FGF2 via maleimide-thiol reaction

2.2.1 Desalt engineered FGF2 containing N-terminal KCKSGG and C-terminal LPETGG sequence to the Reaction Buffer (25 mM HEPES pH 7.0, 10 mM $(NH_4)_2SO_4$, 10 mM methionine, 0.1 mM EDTA) using a column with Sephadex G-25 resin. Adjust the protein concentration to 25 µM with the Reaction Buffer.

2.2.2 Dissolve MMAE in DMAc to prepare 40 mM stock solution.

2.2.3. To the new reaction tube add first four-fold molar excess of MMAE over protein -SH groups. Then add double volume of PBS buffer in relation to the volume of the FGF2 protein. At last add FGF2 protein.

NOTE: In this step order of reagents addition is important.

2.2.4. Carry out the reaction for 1 h at 20 °C with gentle rotation.

2.2.5. Load the reaction mixture on a Carboxymethyl (CM)-Sepharose column and wash off the excess of the unconjugated cytotoxic agent with 25 mM HEPES pH 7.4. These mild conditions prevent protein denaturation.

2.2.6. Elute the MMAE.FGF2 conjugate from the column with 25 mM HEPES pH 7.4 with 0.5 M NaCl.

NOTE: The protocol can be paused here.

2.2.7. Analyze the efficiency of the conjugation using SDS-PAGE. Use 15% separation gel, 10 – 250 kDa protein marker, and analyze bands between 15 and 25 kDa (**Figure 3**).

2.3. Preparation of dual warhead conjugate of enginnered FGF2 using combination of maleimide-thiol chemistry and sortase A-mediated ligation

NOTE: For the generation of dual warhead conjugates of engineered FGF2 steps 2.1 and 2.2 are combined. First MMAE.FGF2 conjugate is produced via maleimide-thiol chemistry (step 2.2) and

used for the attachment of GGGG-MTX with sortase A to the C terminal LPETGG tag of MMAE.FGF2 (step 2.1). Also, reverse procedure is also possible (first sortase A-mediated conjugation, then maleimide-thiol reaction).

2.3.1. Use steps 2.2.1-2.2.7 for the preparation of mono-substituted MMAE.FGF2 conjugate.

2.3.2. Exchange the buffer for the sortase A Reaction Buffer (25 mM HEPES pH 7.4, 150 mM NaCl, 10 mM $(NH_4)_2SO_4$, 5 mM $CaCl_2$) using a column with Sephadex G-25 resin.

2.3.3. Continue as described in 2.1.2-2.1.7.

REPRESENTATIVE RESULTS:

The presented protocols describe two distinct strategies for the conjugation of different cytotoxic drugs into proteins of interest. Furthermore, a combination of individual strategies is shown that allows to generate dual warhead cytotoxic conjugates in a site-specific manner.

As shown on SDS-PAGE gels in **Figure 2** (lane 2 vs. 3) the maleimide-thiol reaction allows to reach almost 100% efficiency for the MMAE conjugation to the Fc fragment (visible as a shift of the Fc band due to the increased molecular weight resulting from MMAE incorporation). Since the Fc fragment has more than one putative conjugation site, we cannot fully exclude the possibility of mixture of mono- and dually-conjugated Fc in lane 3. To solve this point obtained conjugate can be further verified with mass spectrometry, or spectrophotometrically to determine drug to protein ratio, as described in Chen's protocol[20].

High efficiency of the conjugation is also obtained for engineered FGF2 variant using above-described protocols (**Figure 3**). The yield of sortase A-mediated ligation of MTX to the C-terminal LPETGG tag in the engineered FGF2 is close to 100% as estimated from SDS-PAGE (**Figure 3**, lane 3). Similarly, high efficiency of MMAE attachment to the N-terminal KCKSGG sequence of the engineered FGF2 is obtained via maleimide-thiol reaction (**Figure 3**, lane 4). When two reactions are combined, a dual warhead FGF2 conjugate (MMAE.FGF2.MTX) with MMAE and MTX attached in a site specific manner to the N- and C- termini of engineered FGF2, respectively is generated with almost 100% yield (**Figure 3,** lane 5).

FIGURE LEGENDS

Figure 1: Strategies for site-specific conjugation of cytotoxic drugs to the proteins of interest. (**A**) Structures of drugs used for conjugation**.** (**B**) Conjugation of MMAE to the cysteine residues of the Fc fragment of human IgG1. Two disulfide linkages are initially reduced Cysteine residues are first reduced with TCEP to generate reactive thiol groups (SH) that are subsequently conjugated to the maleimide-MMAE. The dimeric assembly of the Fc is retained by the interaction between the constant heavy chain domains 2 and 3 (CH2 and CH3). (**C**) Development of single and dual warhead conjugate of engineered FGF2. Sortase A mediates attachment of the peptide-linked MTX to the C-terminal small LPETGG tag within FGF2 to yield FGF2.MTX. A maleimide-thiol reaction allows for the incorporation of the MMAE to the N-terminal KCKSGG

sequence in engineered FGF2, resulting in MMAE.FGF2. Sortase A reaction allows for generation of MMAE.FGF2.MTX from MMAE.FGF2 and GGGG-MTX

Figure 2: SDS-PAGE of the Fc fragment. SDS-PAGE of the Fc fragment before (lane 2) and after the conjugation with MMAE (lane 3). Lane 1 is a molecular weight marker.

Figure 3: SDS-PAGE of the engineered FGF2. SDS-PAGE of the engineered FGF2 before (lane 2) and after the conjugation with MTX (lane 3), MMAE (lane 4) and MTX+MMAE (lane 5). Lane 1 is a molecular weight marker.

DISCUSSION:

Due to the high interest in the design of selective therapeutics against diverse cancer types there is an urgent need for strategies allowing for site-specific attachment of distinct cargoes to the targeting proteins. The site-specific modification of targeting proteins is critical as it ensures homogeneity of developed bioactive conjugates, a prerequisite for modern therapeutics. There are several methods, both chemical and enzymatic allowing for site-specific attachment of cargo to the protein of choice. In most cases these approaches require protein engineering steps prior to the generation of conjugate of interest. Depending on the selected method the preparation of protein might be laborious and expensive.

Here we provide a protocol in which two distinct strategies for site specific modification of proteins (one example of the Fc fragment of IgG1 and engineered FGF2) using chemical (maleimide-thiol chemistry) and enzymatic (protein ligation with sortase A) approaches are shown that highly efficient and relatively fast. The Fc fragment is a core component of therapeutic antibodies in the ADC approach, while engineered FGF2 is a modified growth factor that specifically targets FGFRs. Furthermore, we provide a protocol how two distinct strategies of conjugation can be combined to yield targeting protein with incorporated two distinct cargoes (in this case different cytotoxic drugs – MMAE and MTX).

A critical step in all presented approaches is the preparation of targeting protein. The protein should be highly pure (to ensure homogeneity of products), properly folded (to ensure biological activity) and often modified prior the conjugation (cysteine engineering, addition of small tags etc.). For homogeneity and site-specificity protein should be engineered to contain a single cysteine residue that is accessible for maleimide-thiol reaction. One possibility is to use naturally occurring surface-exposed cysteine within protein of interest with simultaneous mutation of other potentially reactive cysteines. Importantly, in contrast to coupling to the primary amines (α-amine group at the N-terminus of protein and the ϵ-amine group of lysine residues), conjugation to the cysteine residues does not change the net charge of a protein. If targeting protein lacks surface-exposed cysteine one can introduce this residue by site-directed mutagenesis, replacing amino acids non-essential for biological function (e.g., receptor binding), or for protein folding and stability. Exposed residues located in loops or unstructured regions should be preferentially used for cysteine substitution if the structural information is available for the modified protein. Alternatively, a small tag bearing cysteine flanked by basic residues for improved reactivity can be incorporated either at protein N or C termini. This approach was

already employed with success for generation of cytotoxic FGF1 and FGF2 conjugates[14,15].

Although approaches presented in these protocols require protein engineering steps, these modifications usually have no/little effect on targeting protein stability and biological activity. Furthermore, in contrast to laborious, expensive, and low-efficient incorporation of unnatural amino acids, these protein engineering steps still allow to achieve high yields of pure recombinant proteins.

The other critical step is the maleimide-thiol-based conjugation is efficient reduction of cysteine residues. In contrast, the sortase A-mediated ligation does not require modification of protein core, but it is strictly dependent on the availability of the C-terminal LPETGG peptide. Therefore, the C-terminal small tag should be incorporated into the protein of interest and if required, a linker between the protein core and the small tag should be introduced to ensure accessibility of sortase A to the LPETGG sequence. Moreover, sortase A-based method, in contrast to other ones (i.e., formylglycine-generating enzymes, transglutaminase, tubulin tyrosine ligase, trypsiligase, phosphopantetheinyl transferase or biotin ligase) is highly efficient, established amide bond is stable, reaction conducts in mild conditions, and do not require long or hydrophobic tags[21].

Importantly, in some instances the conjugation can affect stability, structure, or function of the targeting protein. Therefore, it is critical to test the functionality of targeting protein in obtained conjugate (e.g., by analyzing receptor binding or receptor-mediated endocytosis) and its stability (e.g., by measuring the levels of conjugates in serum or cell-conditioned media in time)[22–24].

The provided protocols were successfully used to develop conjugates with FGF1, FGF2 and antibody fragment-Fc as targeting molecules[3,11,12,14,25–27]. However, they can be, in principle, applied to any targeting factor of interest, including the Fc fragment-containing proteins like antibodies, antibody fragments or peptibodies. Presented methods can also be used in combination with other drugs to yield cytotoxic conjugates of proteins for anti-cancer therapies. Alternatively, these protocols can employ fluorophores to develop specific protein-based probes for the diagnostics.

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DISCLOSURES:
The authors have nothing to disclose.

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26. Krzyscik, M.A., Zakrzewska, M., Otlewski, J. Site-specific, stoichiometric-controlled, PEGylated conjugates of fibroblast growth factor 2 (FGF2) with hydrophilic auristatin Y for highly selective killing of cancer cells overproducing fibroblast growth factor receptor 1 (FGFR1). *Molecular Pharmaceutics*. **17** (7), 2734–2748 (2020).
27. Borek, A., Sokolowska-Wedzina, A., Chodaczek, G., Otlewski, J. Generation of high-affinity, internalizing anti-FGFR2 single-chain variable antibody fragment fused with Fc for targeting gastrointestinal cancers. *PLOS ONE*. **13** (2), e0192194 (2018).

Figure 1

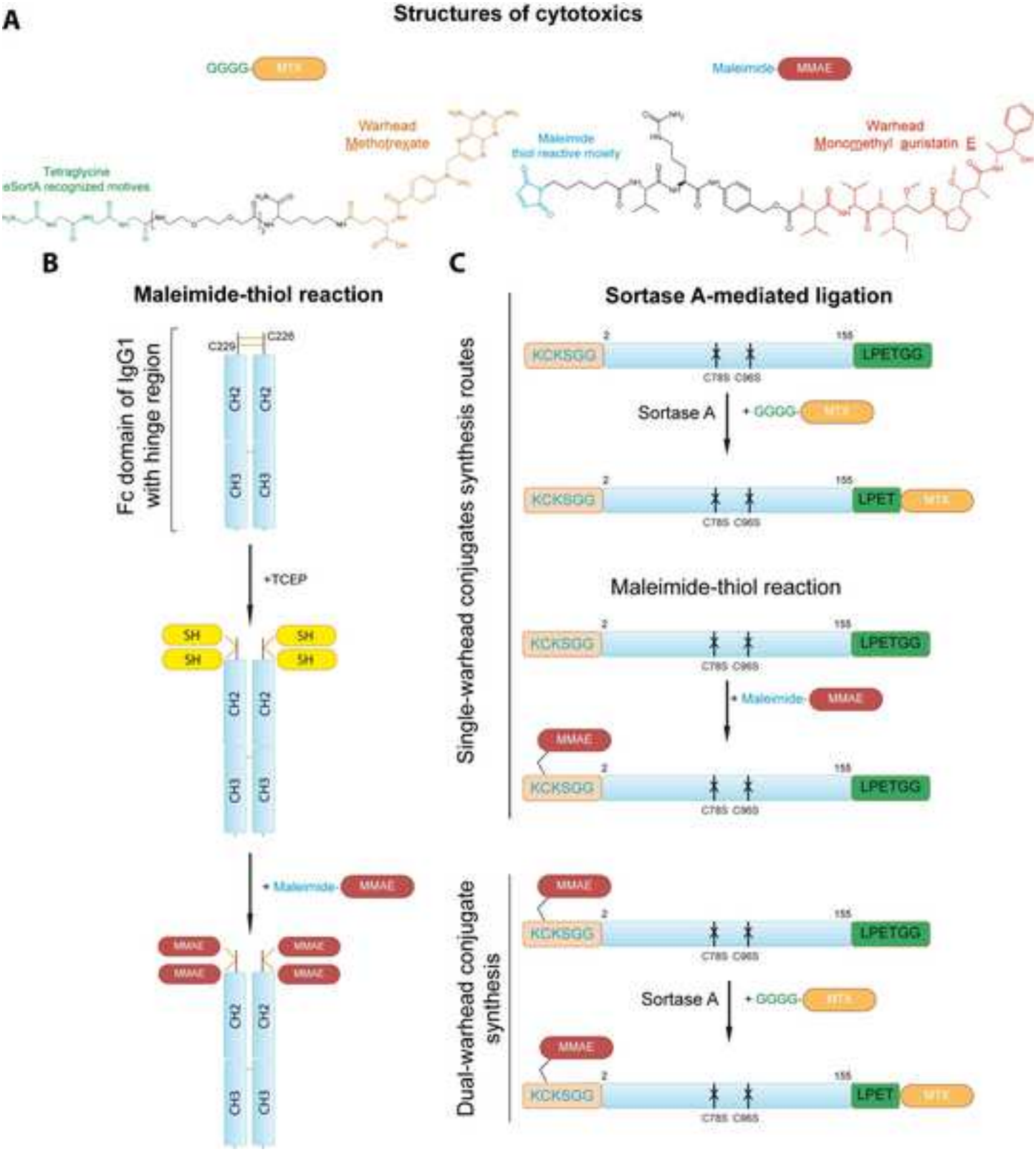
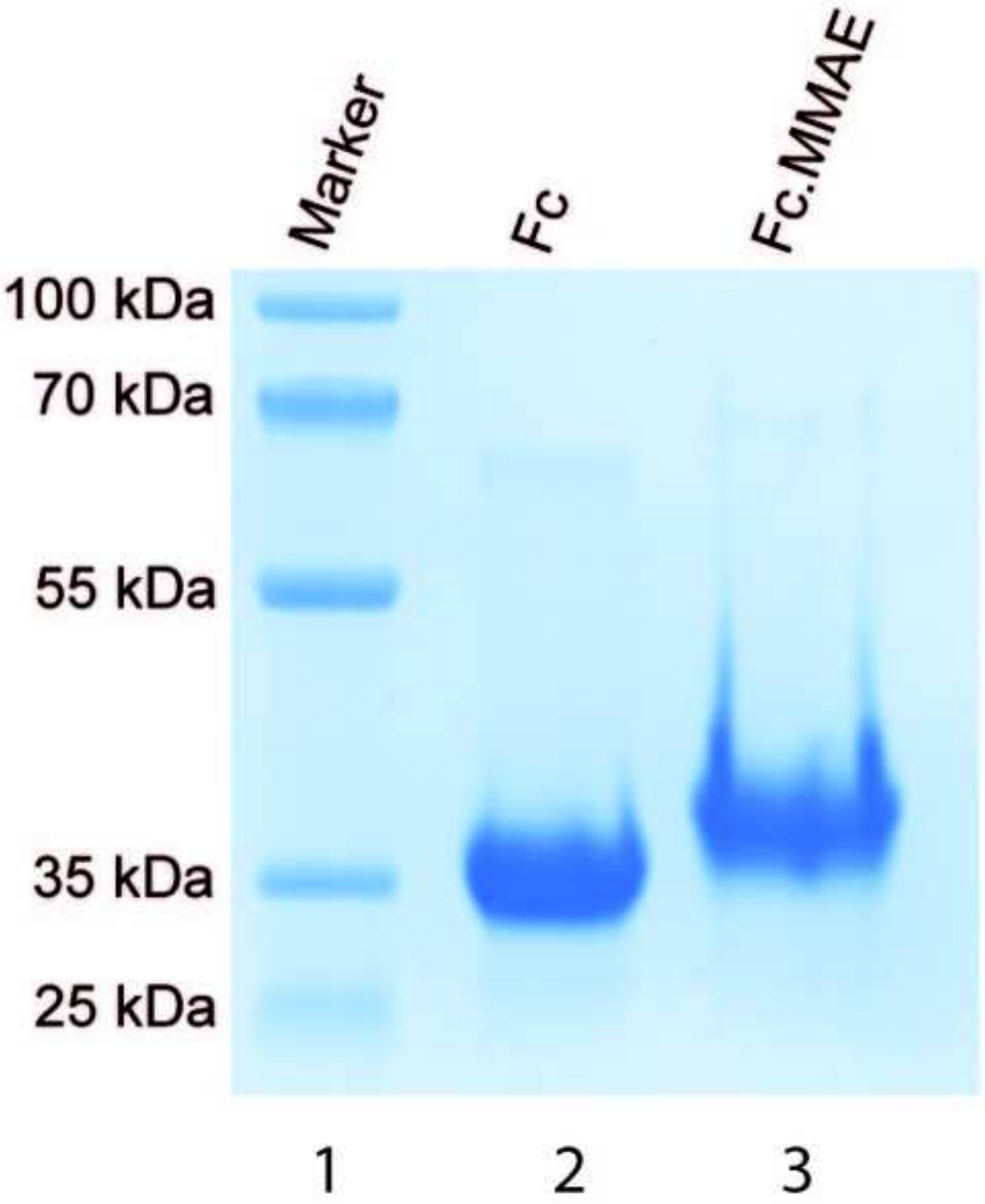


Figure 2



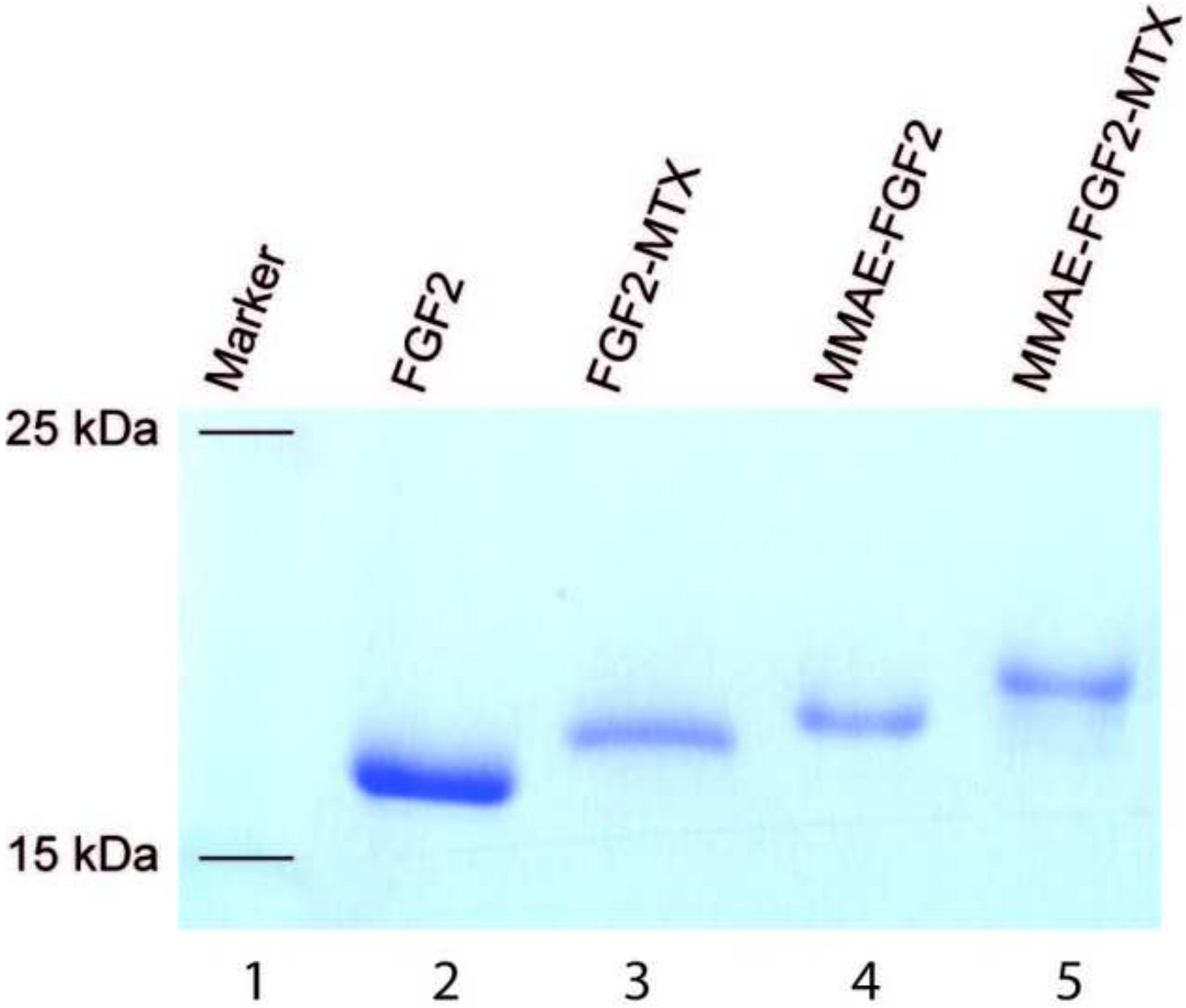


Table of materials

Click here to access/download;Table of Materials;Kopia JoVE_Table_of_Materials-2.xlsx

Name of Material/Equipment	Company
CM-Sepharose column	Sigma-Aldrich, Saint Louis, MO, USA
Heparin Sepharose column	GE Healthcare, Chicago, IL, USA
HiTrap Desalting column	GE Healthcare, Chicago, IL, USA
HiTrap MabSelect SuRe column	GE Healthcare, Chicago, IL, USA
maleimidocaproyl-Val-Cit-PABC-monomethyl auristatin E (MMAE)	MedChemExpress, Monmouth Junction, NJ, USA
N,N -Dimethylacetamide (DMAc)	Sigma-Aldrich, Saint Louis, MO, USA
Tris(2-carboxyethyl)phosphine (TCEP)	Sigma-Aldrich, Saint Louis, MO, USA

Catalog Number	Comments/Description
CCF100	
GE17-0407-01	
GE17-1408-01	
GE11-0034-93	
HY-100374	Toxic
185884	
646547	

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define abbreviations such as DMAc at first use.

The manuscript has undergone thorough proofreading and the abbreviations were explained.

2. Please provide an email address for each author.

E-mail addresses have been provided, as requested.

3. Introduction: As one of your strategies involves sortase A-mediated ligation, besides citing the reference, please explain briefly how ligation of MTX-linked tetra glycine peptide occurs to C-terminal LPETGG sequence.

The reaction has been described in the Introduction section, lines 149-154.

4. Introduction: Please describe briefly when your methods would be appropriate for readers to apply for their own purposes.

The introduction section has been expanded, as requested (lines 157-161)

5. Please remove the embedded figures from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.

The figures have been deleted from the manuscript and provided separately, as requested.

6. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: MedChemExpress, Monmouth Junction, NJ, USA; Sigma-Aldrich, Saint Louis, MO, USA; Addgene (#75144); HiTrap MabSelect SuRe column; Eppendorf tubes; HiTrap Desalting column\

Commercial language has been removed.

7. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Protocols have been updated.

8. Protocol lines 175-177: please cite a reference for the solid phase synthesis of GGG-MTX.

Reference has been included.

9. 1.2: Please use period as decimal, not comma.

Text has been corrected.

10. 1.6, 1.10, 2.1.7, 2.2.7: please indicate clearly what % SDS-PAGE gels should be used (you can also cite a reference), what molecular markers to use, and which bands to look for. Please cite Figures 2 and 3 at the appropriate instances.

Description of SDS-PAGE has been updated.

11. 2.1.3, 2.2.4: Do you agitate these reactions in any way?

The protocol was updated with the information about agitation conditions.

12. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Parts of the protocol selected for video have been highlighted.

13. As we are a methods journal, please revise the discussion (you could delete some of the background information in the first paragraph that repeats information in the introduction) to add modifications and troubleshooting of the techniques, limitations, and significance with respect to other existing methods.

Discussion has been revised.

14. Please sort the Materials Table alphabetically by the name of the material.

Materials Table has been updated.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript reported the detailed protocols for the site-specific incorporation of cytotoxic warheads into targeting proteins using a chemical method employing maleimide-thiol chemistry and an enzymatic approach that relies on sortase A-mediated ligation.

Major Concerns:

1. The authors should discuss how to choose the cysteine mutation site for conjugation.

The significance of cysteine selection for effective site-specific conjugation of targeting protein with the cytotoxic drug was already highlighted in the Discussion section, lines **303-311**. Now, we have extended these paragraph by adding the information how to choose cysteine mutation site for conjugation of proteins lacking surface-exposed cysteines, as requested by the Reviewer.

2. The authors should also provide a way to optimize the ligation efficiency of Sortase A (e.g. how to choose the linker length and how to optimize the enzyme concentration).

We thank the Reviewer for this point. The concentrations of reagents to be used are largely determined by the yield of conjugate to be achieved and the biochemical characteristics of the targeting protein (protein purification effectiveness, its solubility and stability). For effective Sortase A reaction one should use as a starting point 10-100 times lover concentration of Sortase A than the LPETGG-containing target protein and 100x higher concentration of GGGG-MTX than LPETGG-tagged protein. We have now included this information in the protocol.

Minor Concerns:

N/A

Reviewer #2:

Manuscript Summary:

The reported protocol is of general interest and suitable for publication in JoVE. However, major revisions are required to fulfil journal requirements: a more detailed step-by-step protocol, a discussion more focused on the pro and con of the reported protocol and perhaps a protocol limited to either the preparation of single conjugate or a unique conjugation method (Editor's final decision)

Major Concerns:

1/ In my opinion the protocol suffers from dispersion. The authors describe the preparation of two distinct conjugates (perhaps with the aim to demonstrate the broad scope of the reported protocol).

We thank the Reviewer for this point. Indeed we provided protocols for preparation of conjugates using chemical and enzymatic approaches and their combination to yield double-warhead conjugate. We provided examples of how these protocols can be successful applied to two distinct targeting proteins to demonstrate the broad scope of the reported protocol, as pointed out by the Reviewer.

However, they also use two types of conjugation either alone or in combination: an enzymatic ligation and the thio/maleimide chemistry. When thio/maleimide is considered, two different approaches is carried out. In first instance, the authors point out that it is mandatory to add the sulfhydryl-functionalized Fc protein to the maleimide moiety in solution (perhaps because several thio groups have to be conjugated) while a reverse procedure is adopted when coupling the auristatin E to the fibroblast growth factor 2 (compare steps 1.3. and 2.2.3.) Hence the two examples are treated separately while I think that these apparent discrepancies should be discussed.

We apologize for the inconsistency regarding the order of reagents in steps 1.3 and 2.2.3. The approach described in 1.3 is correct; step 2.2.3 was corrected, accordingly.

In which extent does the protocol be respected by someone interested in reproducing a related conjugate preparation?

In principle, provided conjugation protocols can be successfully applied to any engineered targeting protein of interest to generate selective cytotoxic conjugates. This information can be found in the introduction section, lines 149-150 and in discussion section, lines 331-336.

I leave the editor free to decide whether the authors should keep the initial content of the manuscript or focus on either the conjugation based on the maleimide/thio chemistry or perhaps simply on the double warhead-conjugated protein preparation.

2/ The purpose of the study in the context of cancer is well described. However, advantages or limitations of their method over other proposed approaches - which have been correctly listed - is not really detailed (both abstract and main text). For example, it is mentioned that most developed strategies relies on engineering steps carried out on the protein. (Page 8 lines 291-293). It seems that the authors must introduce a tag on their targeting protein meaning that they also have to perform protein engineering. What are the advantages of their approach?

We have discussed the necessity of protein engineering steps in the approach presented in our protocols. Additionally, we have provided some advantages of described protocols over other conjugation methods (Discussion section, lines 324-328).

On the other hand, I can clearly see that controlled introduction of not one but two warheads require the combination of two orthogonal conjugation steps which is certainly not so obvious. Would it be possible to proceed first by carrying out the sortase-catalyzed step and then the thio/maleimide coupling - not discussed in the protocol.

We thank the Reviewer for bringing out this important point. The reverse procedure is also possible. We included this information in the revised protocol, lines 260-261.

3/ Along this line the authors have omitted to really discussed the critical steps of their method. Rather they insist on the necessity to work with highly pure reactants. However, this point is not addressed in the current protocol since the description of the protocol begins with the conjugation steps. If purity is key in the success of the conjugation this aspect should be included.

We agree with the Reviewer that the critical point of our procedure is the preparation of highly pure engineered targeting protein. Since we used two distinct proteins that largely differ in the expression and purification conditions (Fc fragment is produced in mammalian expression system and purified by Protein-A affinity chromatography, while FGF2 is produced in bacterial expression system and purified via heparin-affinity chromatography), and the protocols for production of these recombinant proteins are extensive and already published we decided not to include them in the conjugation protocol. However, for clarity we provided references to the work that describes in detail procedures for isolation of recombinant Fc and FGF2.

4/ The authors provide gel electrophoreses to prove the efficiency of the conjugation. Nice shifts between the different intermediates are observed in Figure 3, an observation which seems to be in agreement with claimed results. However considering Figure 1, it seems difficult to ascertain that

band in lane 3 does not correspond to a mixture of the two mono- and the di-conjugated Fc fragments. Could the authors provide a mass spectroscopy of the product?

We thank the Reviewer for this point. Since the Fc fragment has more than one putative conjugation site we cannot fully exclude the possibility of mixture of mono- and dually-conjugated Fc in lane 3. To solve this point it is possible to apply either mass spectrometry (unfortunately we do not have MS data for this preparation) or to measure drug to protein ratio. We have provided this information in the Results section and included references to our previous work where we showed MS data for conjugates and described protocol for measurements of drug to protein ratio.

5/ The protocol as it stands is not precise enough to make it reproducible. I suppose that when the video will be recorded, the authors will work with a given amount of a protein. I think that the selected scale should serve as the basis for the description of the protocol. That will be useful to determine, for example, which amount of resin for affinity purifications have been used, which volume of eluent…

We have updated the revised protocol with the information about the scale of targeting protein used for conjugation (lines 170-171), as requested by the Reviewer.

Other examples: Step 1.1. "pH adjusted to 6.0…" What solution is used to do that?

We have corrected this inconsistency.

Conditions for SDS PAGE are not given…

We have provided appropriate reference for SDS PAGE, as requested by the Reviewer.

Minor Concerns:

P1 L65 monomethyl

P2 L122 e.g. ; glycosyltransferase

P2 L127 As exemplary proteins

P3 L136 "…four cysteine residues of the Fc are initially reduced…" : two disulfide linkages are initially reduced? (also in Figure 1 caption)

We have corrected the text, as suggested by the Reviewer.

Structures of both activated auristatin and methotrexate are not supplied.

We have included structures of MMAE and methotrexate as new panel A in the revised Figure 1.

What means PABC (P5 L170) or CM-Sepharose (P7 L238)

We have expanded the abbreviations.

P5 L174 Methotrexate?

Please correct throughout the manuscript 0,2 µM or 0,1M into 0.2 µM, 0.1 M…

P5 L195 "the reaction MIXTURE stored…"

P5 L197 …precipitation…

Step 2.2.1. not clear

P9 L299 …provide a protocol in which two distinct strategies…

We have corrected the text, as suggested by the Reviewer.

Reviewer #3:

Manuscript Summary:

Krzyscik and colleagues describe a fairly robust protocol for the site-specific conjugation of cytotoxic drugs to protein targets. The protocol is well presented and comprehensive.

Minor Concerns:

- Although maleimide chemistry is robust, it requires previous protein engineering which could be limiting, depending on the protein target. Within this scope, how the authors ensure that cross-reactivity with lysines is not happening?

Reactions with maleimide are specific for thiols over a pH range of 6.5 to 7.5. At pH 7.0, the reaction of maleimide with sulfhydryls is 1000 times faster than that of primary amines (e.g. α-amine of the N-terminus of protein and an ε-amine group of lysine residues) (Hermanson et al, Bioconjugate Techniques, 2013). At higher pH values there is cross-reactivity with amino groups, but in our protocols, the pH of the buffers does not exceed 7.4.

- this manuscript would benefit from a MS-based analysis of the protein-conjugates to corroborate the SDS-PAGE data.

We agree with the Reviewer that MS-based analysis would verify obtained conjugates, Unfortunately, we do not have MS data for these preparations of conjugates. However, we have provided in the Results section information that MS can be used to confirm conjugation and included references to our previous work where we showed MS data for conjugates.

- would be interesting to know how the authors would tackle stability and function issues arising from the application of this protocol

We thank the Reviewer for this important point. Indeed the conjugation can affect in some cases targeting protein structure and function. To verify the functionality of the targeting protein within the conjugate the functional tests should be performed (e.g. confirmation of receptor binding, measurements of receptor-mediated endocytosis). Furthermore, the stability test should be performed, for example by analyzing conjugate levels in cell conditioned media or in serum using western blotting. We have updated the discussion section accordingly and provided appropriate references to our work where stability and functionality of such conjugates were experimentally verified.