Chapter

Lysine bioconjugation on native albumin with a sulfonyl acrylate reagent

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Abstract. This protocol details a novel bioconjugation strategy that uses a methanesulfonyl acrylate reagent that is directed to the most reactive lysine on human serum albumin, which enables the construction of chemically defined and stable bioconjugates. The reaction proceeds rapidly and a regioselective modification is achieved using a single molar equivalent of the reagent under biocompatible conditions (37 ºC, pH 8.0). Importantly, the bioconjugate retains both the secondary structural content and function of the unmodified protein. During the reaction of the amino group of lysine and the sulfonyl acrylate reagent, methanesulfinic acid is released after the conjugate addition, which then generates an electrophilic acrylate moiety on the protein. This acrylate can be further used for site-specific protein labelling using a synthetic molecule bearing a reactive amine under biocompatible conditions (21 ºC, pH 8.0).

Running title. Lysine bioconjugation with sulfonyl acrylates

Key words. Lysine; sulfonyl acrylate; bioconjugation; aza-Michael addition; human serum albumin.

1. Introduction

Protein-drug conjugates have been reported and evaluated in clinical trials.[1,2] Compared to the parental small molecule drugs, protein-drug conjugates offer several advantages, including half-life extension, localization to a target tissue, avoidance of drug-drug interactions, and toxicity reduction.[3,4,5] Site- or residue-specific methods to install drugs on proteins simplify the interpretation of results and yield predictable conjugates.[6] However, chemical reactions that can proceed under conditions mild enough to maintain the structure and function of the modified proteins are limited. Even more rare are chemical strategies that can target a single site, leading to products with uniform properties and optimal function without the need for sequence engineering.[7]

Conjugation to serum albumin (HSA) has emerged as a powerful approach for adjuvating the immune response triggered by synthetic vaccines and extending the *in vivo* half-life of many small molecule and peptide/protein drugs.[8,9] However, HSA conjugation strategies, can often yield heterogeneous mixtures with inadequate pharmacokinetics, low efficacies, and variable safety profiles. To date, none of these compounds have displayed site specificity for a single lysine (site-selective bioconjugation). Barbas *et al*. first approached this goal in 2014 by showing that certain lysine residues in albumin reacted more rapidly with α,β-unsaturated sulfonamides than other lysine residues.[10]

The method reported therein explores a two-step strategy to build protein-drug conjugates: the first step consists of lysine chemo- and regioselective chemical modification utilizing a methanesulfonyl acrylate aza-Michael acceptor; the second step then generates a type-2 alkene that can be further modified through a second aza-Michael ligation with amine- and hydroxylamine-containing molecules (**Fig. 1**).[11] Many relevant conjugation reagents such as fluorescent probes, polyethylene glycol (PEG), carbohydrate, deoxyribonucleic acid (DNA) or drug derivatives are commercially available and feature a free reactive amine handle. This two-step, irreversible and chemoselective approach can be directly used to modify several commercial proteins and antibodies bearing free lysine residues (can be confirmed by protein digestion and analysis *via* LC–MS/MS analysis – see subheading 3.3), and in some cases even in the presence

of potentially reactive cysteines. In this protocol, we describe the method for the modification of recombinant human serum albumin (rHSA) at a single lysine from potential 59 reactive lysines.

[Fig 1 here]

2. Materials

Prepare all reagents and solutions using ultrapure water (prepared by purifying deionized water at 25 ºC to >15 MΩ/cm resistance and filtered through a 0.2-µm disc filter) and analytical grade reagents. Prepare all reagents at room temperature and store them at 4 ºC (unless indicated otherwise). Use all commercially available reagents as received unless otherwise noted. Reagents used in the procedure are potentially dangerous, and appropriate care should be taken during their manipulation. The solid and liquid waste products generated should be disposed of appropriately, as defined locally.

2.1 Sulfonyl acrylate reagent

1. Methyl 2-(bromomethyl)acrylate 97%.
2. Methanol anhydrous, 99.8%.
3. Sodium methanesulfinate technical grade, 85%.
4. Ethyl acetate (distilled under CaH_2).
5. Hexane (distilled under CaH_2).
6. Sodium chloride saturated solution.
7. Magnesium sulfate anhydrous, ≥ 98.0%.
8. Sulfonyl acrylate reagent characterization:
    - 8.1. Potassium permanganate ($KMnO_4$) staining solution for thin layer chromatography (TLC).
    - 8.2. Silica gel high purity grade, Merck grade 9385 pore size 60Å, 230-400 mesh particle size for flash column chromatography (5 cm diameter column).
    - 8.3. Deuterated chloroform ($CDCl_3$) for nuclear magnetic resonance (^1H NMR and ^{13}C RMN).

8.4. Mobile phases for liquid chromatography–mass spectrometry (LC-MS): 95% aqueous acetonitrile with 0.05% formic acid and 10 mM ammonium acetate with 0.1% formic acid; 50% aqueous acetonitrile with 0.25% formic acid.

2.2 Reagents for protein modification

1. rHSA was kindly provided by Albumedix Limited. rHSA (59 lysines, 1 free cysteine, 17 disulfide bonds).
2. Methyl 2-((methylsulfonyl)methyl)acrylate (**1**, synthesised as described in subheading 3.1).
3. Benzylamine ReagentPlus®, 99%.
4. *N,N*-Dimethylformamide anhydrous (DMF).

2.3 Solutions for protein modification

1. Formic acid, LC-MS grade.
2. Acetonitrile for high-performance liquid chromatography (HPLC ultragradient grade).
3. LC-MS solvent A for protein/antibody analysis: deionized H_2O + 0.1% formic acid.
4. LC-MS solvent B for protein/antibody analysis: 71% MeCN, 29% H_2O, 0.075% formic acid.
5. 20 mM Tris-HCl, pH 8.0.
    5.1. Prepare 800 mL of distilled water in a suitable container.
    5.2. Add 2.420 g of Tris base to the solution.
    5.3. Adjust solution to pH 8.0 using HCl (notes 1 and 2).
    5.4. Add distilled water until volume is 1 L.
    5.5. Filtrate under vacuum using Millipore Express® PLUS Membrane Filters (note 3).
6. 50 mM NaPi , pH 7.4.
    6.1. Prepare 800 mL of distilled water in a suitable container.
    6.2. Add 10.105 g of sodium phosphate dibasic (Na_2HPO_4) to the solution.

6.3. Add 1.697 g of sodium phosphate monobasic (NaH_2PO_4) to the solution.

6.4. Adjust solution to final desired pH using HCl or NaOH (note 2).

6.5. Add distilled water until volume is 1 L.

6.6. Filtrate under vacuum using Millipore Express® PLUS Membrane Filters (note 3).

2.4 Equipment

1. Bruker 400 MHz DPX-400 Dual Spectrometer.
2. LC–MS system for sulfonyl acrylate reagent characterization: Waters micromass ZQ instrument equipped, electrospray (ESI) ionization, with Waters 2795 HPLC and a Waters 2996 photodiode array detector. The separation technology is based on a 50x4.6 mm C18 column (currently a Phenomenex Kinetix solid core column).
3. LC–MS system for protein analysis: Xevo G2-S TOF mass spectrometer coupled to an Acquity UPLC system using an Acquity Q6 UPLC BEH300 C4 column (1.7 mm, 2.1 × 50 mm).
4. Chirascan spectrophotometer equipped with a Quantum TC125.
5. Vortex shaker suitable for short-time operation (touch function), activated by pressing shaker attachment or continuous operation.
6. Eppendorf thermomixer shaker incubator – heating/mixing/cooling.

2.5 Consumables

1. Flash-chromatography column available with ball joints (capacity 400 mL, useful for rapid, preparative separations).
2. TLC plates: Merck TLC Silica gel 60 F254 glass plates (cut into rectangles of 5x15 cm).
3. NMR tubes: thin glass walled tubes (5 mm diameter).
4. Mass vials: polypropylene 12 x 32 mm screw neck vial, with cap and preslit PTFE/silicone septa 300 μL volume.
5. Quartz cuvette for circular dichroism: parameter 200-2,500 nm spectral range, pathlength 10 mm, chamber volume 300 μL.

3. Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Synthesis of sulfonyl acrylate reagent

[Scheme 1 here]

1. Prepare a solution of 0.18 mL of methyl 2-(bromomethyl)acrylate (1.5 mmol) in 5 mL of methanol.
2. Treat the solution with 0.18 g of sodium methanesulfinate (1.5 mmol) portion wise over 10 min at room temperature (note 4).
3. Stir the solution at room temperature for a period of 1.25 h and concentrate the solution in vacuum by evaporating all the solvent.
4. The residue is taken up in water (10 mL) and extracted four times with ethyl acetate (4x 10 mL) using a liquid/liquid extraction flask. Liquid–liquid extraction is a very well-known method to separate compounds (usually the desired compound from impurities) based on their relative solubilities in two different immiscible liquids, usually water (polar) and an organic solvent (non-polar).
5. Wash the combined ethyl acetate solution (40 mL) with saturated sodium chloride solution in an Erlenmeyer flask, dry over anhydrous magnesium sulfate, filter (fluted paper filter) and concentrate in vacuum to give a white solid.
6. Purify the solid residue by flash column chromatography (pre-packed until 16 cm high with silica gel – see subheading 2.1) eluting with hexane/ethyl acetate 8:2, to give desired product (**Scheme 1**) as a white solid (0.175 g, 65% yield) (notes 5 and 6).
7. Analyse reaction mixtures by analytical TLC on TLC silica gel plates (see subheading 2.5). Visualization is accomplished with UV light (254 nm) or $KMnO_4$ staining solution.
8. ^{1}H NMR and ^{13}C NMR spectra are recorded on a Bruker 400 MHz DPX-400 Dual Spectrometer in deuterated $CDCl_3$ as a solvent using tetramethylsilane as an internal standard. To prepare the sample, 5 mg of the product are dissolved in deuterated

$CDCl_3$ and transferred to an RMN tube. Chemical shifts are reported in parts per million (ppm) on the δ scale from tetramethylsilane (NMR descriptions: s, singlet).

1. Mass spectroscopy is performed using a Waters micromass ZQ (LC–MS) instrument (see subheding 2.4). This system is an automated service utilizing electrospray (ESI) ionization. To prepare the sample, 1 mg of the product is dissolved in methanol and transferred to a mass vial (see subheading 2.5).
9. The mobile phases are described in subheading 2.1. The separation technology is based on a 50x4.6 mm C18 column (currently a Phenomenex Kinetix solid core column) (note 7). The system runs using 50% aqueous acetonitrile with 0.25% formic acid as mobile phase and can measure accurate masses from 150 Da to 1500 Da.
10. Characterization of compound **1**: Melting point 64.9–66.7 ºC. ^{1}H NMR (400 MHz, $CDCl_3$): 2.89 (s, 3H, CH_3), 3.81 (s, 3H, OCH_3), 4.05 (s, 2H, CH_2), 6.15 (s, 1H, CH_2), 6.62 (s, 1H, CH_2). ^{13}C NMR (100 MHz, $CDCl_3$): 40.5, 52.7, 56.4, 128.8, 134.2, 165.8. DEPT: 40.5, 52.7, 56.4, 134.2. IR (ATR): 1712 (CO) cm^{-1}. LC-MS m/z (ESI): 179 (MH^+). High-resolution mass spectrometry (HRMS) calcd. for $C_6H_{10}O_4S$ (MH^+): 179.0300; found: 179.0347. Data is consistent with that previously reported.[12]

3.2 rHSA conjugation with 1

[Fig 2 here]

1. Prepare a solution of 9.4 µL of 20 mM Tris-HCl, pH 8.0 and 3.3 µL of DMF (note 8) in an eppendorf.
2. Add a 26.6 µL aliquot of a stock solution of 15.05 µM rHSA (notes 9 and 10).
3. Vortex the resulting mixture for 10 seconds (note 11).
4. Add 0.7 µL of a 0.56 mM solution of **1** (1 equiv.) in DMF (**Fig. 2**, notes 12 and 13).
5. Shake the reaction for 1, 2 and 24 h, at 37 ºC (600 rpm).
6. Analyse a 10 µL aliquot at each reaction time by LC–MS (note 14).
7. Complete conversion to the expected product is observed after 1 h (calculated mass, 66536 Da; observed mass, 66536 Da, **Fig. 3**).

8. Further rHSA enzymatic digestion followed by LC–MS/MS analysis enables identification of the modified lysine residue. In the case of rHSA, we found that the modification occurred in the peptide G**K**KLVAASQAALGL (modified residue underlined and in bold, total of 91% sequence coverage) as observed in the MS/MS spectrum, which corresponds to lysine 573.[11]

[Fig 3 here]

3.3 Protein analysis by LC-MS

1. LC–MS is performed on a Xevo G2-S (see subheading 2.4).
2. LC-MS solvents A and B (see subheading 2.3) are used as the mobile phase at a flow rate of 0.2 $mL \cdot min^{-1}$. The gradient is programmed as follows: 72% A to 100% B after 25 min then 100% B for 2 min and after that 72% A for 18 min (note 15).
3. The electrospray source is operated with a capillary voltage of 2.0 kV and a cone voltage of 40 V.
4. Nitrogen is used as the desolvation gas at a total flow of 850 $L \cdot h^{-1}$.
5. Mass vials described in section 2.5 are used for the measurements.
6. Total mass spectra are reconstructed from the ion series using the MaxEnt algorithm preinstalled on MassLynx software (v. 4.1 from Waters) according to the manufacturer's instructions.
7. To obtain the ion series described, the major peak(s) of the chromatogram are selected for integration and further analysis.
8. The total ion chromatogram, combined ion series and deconvoluted spectra are shown for the rHSA (**Fig. 4**). Identical analyses are carried out for all the conjugation reactions performed.

[Fig 4 here]

3.4 Circular dichroism

1. Circular dichroism spectroscopy is used to analyse rHSA and rHSA–**1** secondary structure in solution (**Fig. 5**). The different types of secondary structure –α-helix,

β-sheet and random coil– all have characteristic circular dichroism spectra in the far-UV region of the spectrum (190-250 nm).

2. Concentrate the samples to 10 nM in 50 mM NaPi, pH 7.4 using a Vivaspin® 500, 30,000 MWCO PES (Sartorius) column (note 16).
3. Record circular dichroism measurements using a Chirascan spectrophotometer equipped with a Quantum TC125 temperature control unit (25 ºC).
4. Acquire the data in a 0.1 cm path length with a response time of 1 s, a per-point acquisition delay of 5 ms and a pre- and post-scan delay of 50 ms.
5. Spectra are an averaged over three scans, in a wavelength range from 200 nm to 260 nm, and the spectrum from a blank sample containing only buffer was subtracted from the averaged data.
6. Circular dichroism analysis of rHSA and rHSA−**1** showed no alterations in secondary structural content, which reflects the mildness and efficiency of the conjugation process.

[Fig 5 here]

3.5 rHSA–1 conjugation with benzylamine

[Fig 6 here]

1. Thaw a 20 μL aliquot of rHSA–**1** (10 μM) in 20 mM Tris-HCl, pH 8.0 buffer (notes 17 and 18).
2. Add 0.2 μL of 9.5 M solution of benzylamine (9500 equiv., in DMF) (**Fig. 6**, note 19).
3. Vortex the resulting mixture for 10 seconds.
4. Shake the mixture for an additional hour at 21 ºC (600 rpm).
5. Remove small molecules from the reaction mixture by loading the sample onto a Zeba Spin Desalting Column previously equilibrated with 20 mM Tris-HCl, pH 8.0 buffer (note 20). The sample is eluted via centrifugation for 2 min at 1500 *g*.
6. Analyse a 10 μL aliquot by UPLC-MS (described in subheading 3.2).

7. Complete conversion to the expected product is observed after 1 h (calculated mass, 66645; observed mass, 66643, **Fig. 7**).

[Fig 7 here]

4. Notes

1. The Tris-HCl buffer should be strictly 20 mM and pH 8.0. The reaction is very sensitive to the presence of salts, ionic strength and pH. The use of 20 mM Tris-HCl, pH 8.0 buffer is crucial to achieve complete conversion to the elimination product.

2. Concentrated HCl (12 M) can be used at first to narrow the gap from the starting pH to the required pH. From then on it would be better to use a series of HCl (i.e., 6 and 1 M) with lower ionic strengths to avoid a sudden drop in pH below the required pH.

3. When the buffers are not being used every day, consider to storage them at 4 ºC to avoid degradation. Also, take aliquots and avoid using buffers prepared by other users. Once prepared and filtrated, the buffers can be stored and used for up to 1 month at room temperature (15–25 ºC).

4. Sodium methanesulfinate must be added portion wise for the reaction to be complete.

5. Methyl 2-((methylsulfonyl)methyl)acrylate (**1**) can be stored as a solid at –20 ºC for at least 4 months.

6. Toxicity information for this compound is not available: assume that it is toxic. Always handle the compound with gloves and work in an appropriately ventilated environment.

7. There are several methods available enabling the user to produce mass spectra for compounds up to 2k Da in positive and negative modes of ionization. In some cases, a Waters LCT Premier combined with an Agilent 1100 autosampler can also be used.

8. The use of 10% DMF ensures the complete solubility of the reagent.

9. rHSA (100mg/mL stock solution) can be stored at 4 ºC for up to 6 months. Consider running a sample control before starting the experiments. Check the sample concentration before starting the labelling by using for example the Bradford protein assay.

10. The diluted solutions of both protein (15.05 μM) and reagent (0.56 mM) should be fresh (storage for no longer than 24 h, at 4 ºC, before use). Stock solutions (1 M) of this reagent should be freshly prepared on the day of the experiment.

11. Vortex all the solutions for them to be homogeneous.

12. More than one single modification can be observed when using more than 1 equivalent of reagent. Concentrations of stock solutions should be accurate. To avoid errors, prepare a concentrated stock solution (1 M) and take aliquots from it to prepare diluted ones.

13. If the reaction is scaled-up, the linker should be gradually dropped into the reaction solution while shaking. Both reaction and bioconjugate analysis are similar up to 150 μM/500 μL (highest concentration/volume tested).

14. Centrifuging the samples prior to the run helps remove insoluble particles that can damage the HPLC column (2000 *g*, 10 s, room temperature).

15. For best results, freshly prepare all HPLC solvents and purge the LC–MS system before analysis of protein samples. Results can be slightly different based on the equipment.

16. Vivaspin® 500 columns are ideal for small molecules elimination, buffer exchange and concentration of the protein/bioconjugates samples. Follow manufacturer's instructions.

17. For further use of rHSA–**1** conjugate, reagent excess can be removed by loading the sample onto a Zeba spin desalting column, pre-equilibrated with 20 mM Tris-HCl, pH 8.0. Perform the purification eluting the sample following the manufacturer's instructions. Check the sample concentration after this procedure using for example the Bradford protein assay.

18. The rHSA–**1** conjugate can be stored at −20 ºC for up to a month.

19. Conditions must be optimized for different amines. Terminal primary and secondary amines can be used.

20. The huge excess of benzylamine may interfere with the resolution of the LC-MS spectra and also damage the HPLC column. Small molecules should be removed from the reaction mixture following the described method.

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Figure legends:

Scheme 1. Synthesis of **1**.

Figure 2. Typical analysis of rHSA conjugation. The total ion chromatogram, combined ion series and deconvoluted spectra are shown.

Figure 3. Scheme of reaction for the bioconjugation rHSA with **1**.

Figure 4. Combined ion series and deconvoluted mass spectrum of the reaction of rHSA (10 μM) with 1 equiv. of **1** after 1 h at 37 ºC. Identical data is obtained at 2 and 24 h.

Figure 5. Structural analysis of rHSA and rHSA–**1** by circular dichroism.

Figure 6. Scheme of reaction for the bioconjugation rHSA–**1** with benzylamine.

Figure 7. Combined ion series and deconvoluted mass spectrum of the reaction of rHSA–**1** (10 μM) with 9500 equiv. of benzylamine in 20 mM Tris-HCl, pH 8.0 TrisHCl with 10% DMF after 1 h at 21 ºC.