Phosphine-Mediated One-Pot Thiol-Ene "Click" Approach to Polymer-Protein Conjugates

Mathew W. Jones,[a] Giuseppe Mantovani,[b] Sinead M. Ryan,[c] Xuexuan Wang, [c]

David J. Brayden,[c] and David M. Haddleton[a]*

a. Department of Chemistry, University of Warwick, Coventry CV4 7AL, United Kingdom; Fax: +44 24 7652 4112; Tel: +44 24 7652 3256

E-mail: D.M.Haddleton@warwick.ac.uk

b. School of Pharmacy, University of Nottingham, Nottingham NG7 2RD, United Kingdom

c. UCD School of Agriculture, Food Science and Veterinary Medicine and UCD Conway Institute, University College Dublin, Belfield, Dublin 4, Ireland

1.0 General

1.1 Reagents. Salmon calcitonin acetate was purchased from PolyPeptide Laboratories (Hillerod Denmark) and stored at 4 °C. ($mPEG_{475}$)MA and tris(2-carboxyethyl) phosphine (TCEP) were purchased form Sigma-Aldrich and used as received. Acetonitrile (MeCN) far UV employed for HPLC analysis was purchased from Romil. All the other reagents and organic solvents used were at least ACS grade.

1.2 Analysis RP-HPLC was carried out using a Jupiter C_{18} (5 micron) 250 x 4.6 mm column. The HPLC system comprised of two Gilson 306 pumps a Gilson 811B mixer and a Gilson 805 manometric module; the sample was injected using a SPARK Endurance autosampler. Sample detection was carried out using two UV detectors connected in series, a Jasco-975 and Knauer K-2001 monitored at λ = 215 nm and λ = 280 nm respectively (unless otherwise stated). The mobile phases used were: a) mobile phase A: 90 % v/v water, 10 % v/v MeCN (far UV) and 0.05 % v/v TFA; b) mobile phase B: 100 % v/v MeCN (far UV) and 0.04 % v/v TFA. The column was equilibrated for 10 minutes by washing with mobile phase A before sample injection. To ensure that the column was thoroughly washed before each sample injection and to prevent the build up of contaminants, the gradient included a final washing step whereby the concentration of mobile phase B was increased to 80 %. Without this washing step a build up of contaminants present in the solvents was observed which had an adverse effect on the quality of the data obtained. HPLC grade solvents/reagents were used in all experiments. The gradient used is given in Table 1.

Time (min)	% Mobile phase A	% Mobile phase B
0	90	10
27	40	60
37	20	80
48	20	80
52	90	10
60	90	10

Table 1. Gradient used for RP-HPLC analysis of the conjugates studied in this work.

LC-MS was carried out using a RP Jupiter C_{18} column (5 micron) 250 x 4.6 mm with the UV traces monitored at λ = 215 nm and at λ = 200 nm. The output from the UV detector passed through a flow splitter before reaching an Esquire 6000 ESI Ion Trap mass spectrometer. The flow rate was 1 mL/min and the injection volume was 20 µL. The mobile phases used were: a) mobile phase A: 100 % water, and 0.1 % formic acid; b) mobile phase B: 100 % MeCN (far UV) and 0.1 % formic acid. The gradient used is described in Table 2.

Time (min)	% Mobile phase A	% Mobile phase B
0	100	0
8	100	0
24	45	55
29	0	100
31	0	100
41	100	0
50	100	0

Table 2. Gradient used for LC-MS analysis of tryptically digested sCT and sCT-$(mPEG_{475})_2$ conjugates.

Mass spectra were acquired by MALDI-ToF (matrix-assisted laser desorption and ionization time-of-flight) mass spectrometry using a Bruker Daltonics Ultraflex II MALDI-ToF mass spectrometer, equipped with a nitrogen laser delivering 2 ns laser pulses at 337 nm with positive ion ToF detection performed using an accelerating voltage of 25 kV.

2. Synthesis of sCT-$(mPEG_{475})_2$ conjugate.

TCEP·HCl (7.1 mg, 25 µmol) was dissolved in water (1.0 mL, 18 MΩ). A 100 µL aliquot (2.5 µmol of TCEP·HCl, 1.7 eq) of this solution was taken and added to a vial containing a solution of salmon calcitonin (5.0 mg, 1.5 µmol, 1.0 eq) in water (0.4 mL, 18 MΩ) and the resulting solution was stirred at ambient temperature. RP-HPLC analysis revealed that quantitative reduction of the Cys^1-Cys^7 disulfide bridge occurred after 30 minutes. Poly(ethylene glycol) methyl ether acrylate (average M_n ~454, 4.84 mg, 10.2 mmol, 7.0 eq) was dissolved in phosphate buffer (0.500 mL, 500 mM, pH 7.0) and the resulting solution was added to the reduced peptide solution and left to stir at ambient temperature.

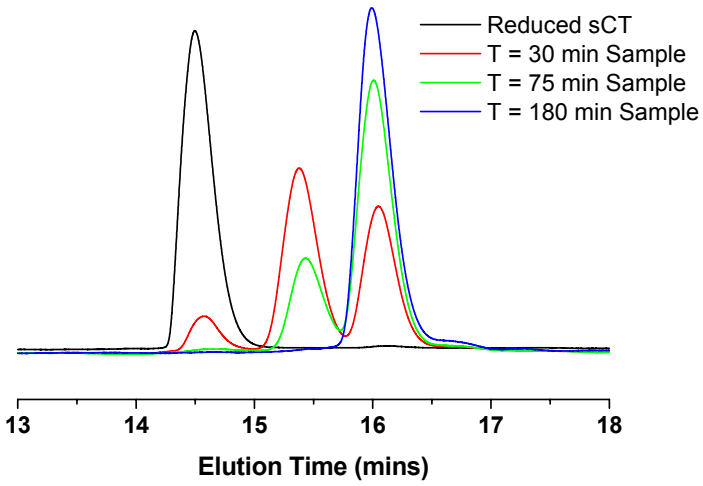


Figure 1. C_{18} RP-HPLC monitoring of the one-pot 2-steps thiol-ene click process.

The bioconjugation reaction was monitored by RP-HPLC and quantitative conversion was observed after 2 hours. The solution was then diluted with water and dialysed (2 kDa MWCO) membrane against pure water for 5 days. The solution was then lyophilised and the conjugate was isolated as a white powder.

0.1 mg of the conjugate was dissolved in 100µL of water and spotted onto a MALDI plate pre-spotted with 2 µL of trifluoroacetic acid (10 mg mL^{-1} in H_2O) and 2 µL of a 2,5-dihydroxybenzoic acid (10 mg mL^{-1}) solution. MALDI-Tof confirmed the presence of the peptide conjugated to two poly(ethylene glycol) chains, with no higher conjugates observed.

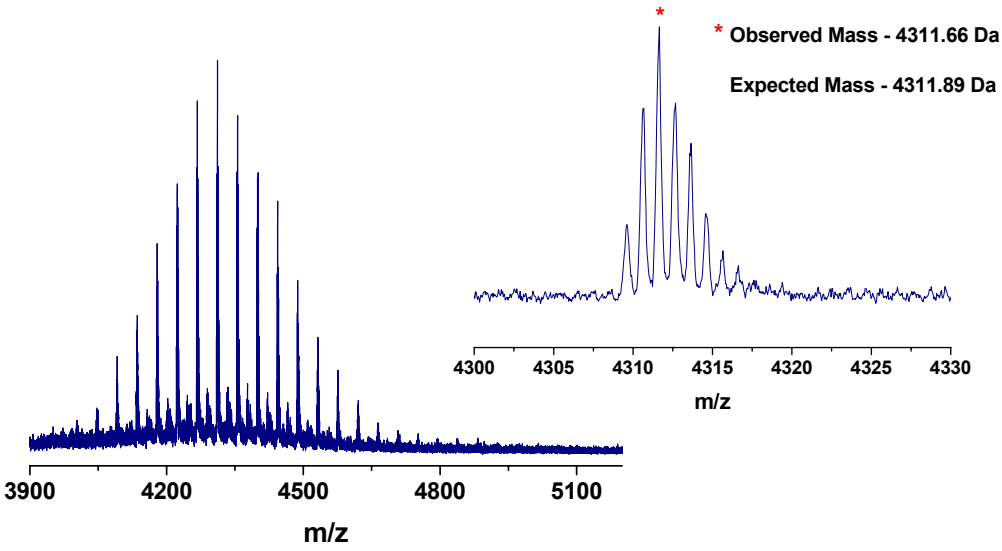


Figure 2. MALDI-ToF spectra of sCT conjugated to two PEG-acrylate chains.

The observed masses for the conjugate are in good accordance with theoretical values, with the predominant distribution corresponding to the protonated conjugate and a second distribution corresponding to the sodiated derivative. No other PEGylated species were observed by MALDI-ToF.

3. Tryptic digestion experiments.

Trypsin digestion of native sCT and the sCT-$(mPEG_{475})_2$ conjugate was performed using a Trypsin Singles™ kit. The conjugate was incubated at 37°C for 14 hours. Following incubation, a sample was taken for MALDI-ToF analysis: 2 µL of the digested conjugate solution was mixed with 2 µL of sodium trifluoroacetate (10 mg mL^{-1} in H_2O) and spotted onto a MALDI plate pre-spotted with 2 µL of a 2,5-dihydroxybenzoic acid (10 mg mL^{-1}) solution.

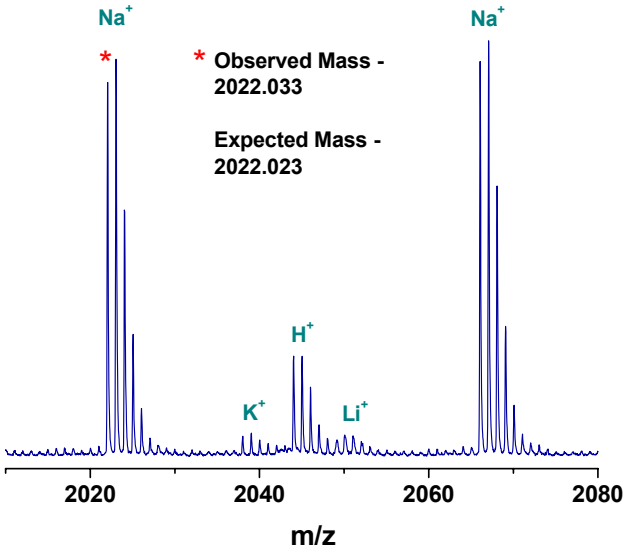


Figure 3. Section of MALDI-ToF spectrum from $(Cys^{1}\text{-}Lys^{11})$-$(mPEG_{475})_2$ region.

Although the predominant distribution of the PEGylated peptide-fragment is the sodiated derivative, protonated, lithiated and potassiated distributions are visible upon closer inspection of the spectrum.

4. Bioactivity assays

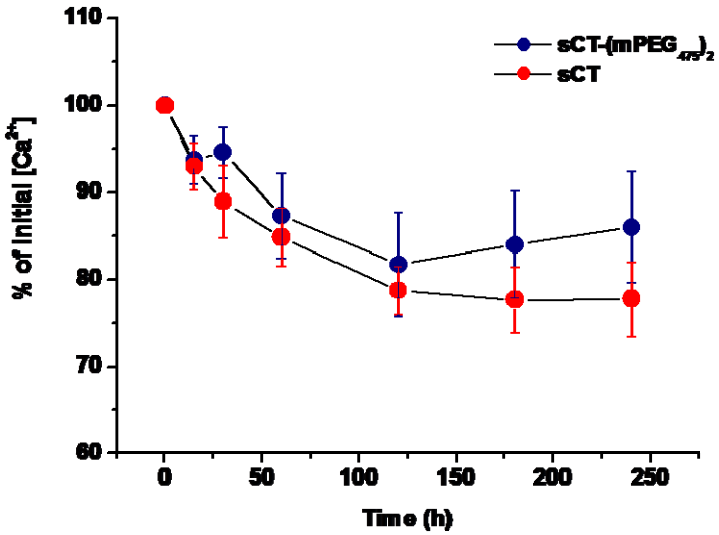
(a) In vitro bioassay.

The capacity of native sCT and the sCT-$(mPEG_{475})_2$ conjugate to increase intracellular cAMP was assessed using a standard ELISA kit in sCT receptor-bearing

cells.[1] T47D human breast cancer cells were grown in RPMI-1640 culture medium containing 1 % penicillin–streptomycin, 10 % fetal bovine serum, and insulin (0.2 IU/mL). Cells were seeded on 24 well plates at a density of 1.0×10^6 cells/well and incubated in 95 % O_2 and 5 % CO_2 at 37 °C for 2 days. After washing with HBSS, cells were pre-incubated with the same medium supplemented with the phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine (IBMX, 0.2 M), at 37 °C for 30 minutes. The cells were then incubated with sCT or sCT-$(mPEG_{475})_2$ conjugate. After removing the supernatant, the intracellular cAMP was extracted from the cells by cell lysis and measured by ELISA (R & D Systems, UK).

(b) In vivo assay

Rats (200-300 g) were intravenously injected via tail vein with different sCT formulations containing 200 IU/mL sCT content at the dose of 1 mL/kg body weight. Both sCT formulations of sCT and sCT-$(mPEG_{500})_2$ had comparable ability in lowering plasma calcium



5. References

1. S. B. Fowler, S. Poon, R. Muff, F. Chiti, C. M. Dobson and J. Zurdo, *Proc. Natl. Acad. Sci. U. S. A.*, 2005, **102**, 10105-10110.