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1 Conjugation of an oligonucleotide to Tat, a cell 2 penetrating peptide, *via* click chemistry

3 Sarah D. Brown and Duncan Graham*

4 *Centre for Molecular Nanometrology, WestCHEM, Department of Pure and Applied*
5 *Chemistry, University of Strathclyde, 295 Cathedral Street, Glasgow, UK, G1 1XL.*

6 *Corresponding author. Tel: +44141 5484701. Fax: +441415520876.

7 Email: duncan.graham@strath.ac.uk

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10 **Abstract:** Uptake of diagnostic and therapeutic oligonucleotides that specifically target
11 disease can be enhanced by attachment of a cell penetrating peptide. Here we describe the
12 covalent attachment of an oligonucleotide to Tat, a biologically important cell-penetrating
13 peptide, *via* click chemistry.

14 **Keywords:** oligonucleotide peptide conjugates, Tat peptide, click chemistry.

15

16 Detection and treatment of disease on a cellular level using oligonucleotides is an elegant
17 strategy with high specificity and low toxicity.¹ Delivery of a nucleic acid sequence into the
18 cell, however, is made difficult by the efficiency of the cell itself. The plasma membrane is a
19 highly effective barrier with a net negative charge, repelling the phosphate backbone of
20 oligonucleotides.² Attachment of cell-penetrating peptides (CPPs) to oligonucleotides is well
21 documented and has been found to facilitate transfection and enhance resistance to
22 degradation of nucleic sequences.³⁻⁹ The conflicting chemistries of peptide and
23 oligonucleotide synthesis make in-line conjugation challenging. Total solid-phase synthesis is
24 overcoming these problems, however, the method is not very flexible.¹⁰ Synthesising the two
25 biomolecules and linking them in solution (fragment conjugation) avoids these problems but
26 can be labour intensive, time-consuming and can generate poor yields. Tat peptide, derived
27 from HIV-1 Tat protein, is a cell-penetrating peptide of biological interest due to its widely
28 reported success in transporting various cargoes into cells.¹¹⁻¹⁵ Tat peptide, however, is
29 notoriously difficult to handle, often precipitating out of reaction mixtures.¹⁶⁻¹⁷ Whilst Gogoi
30 *et al.* have produced oligonucleotide peptide conjugates (OPCs) using click chemistry,¹⁸ we

31 provide the first report of an oligonucleotide Tat peptide conjugate *via* the copper-catalysed
32 azide alkyne cycloaddition (CuAAC). In addition, we have used highly denaturing conditions
33 to ensure that the biomolecules come together covalently rather than electrostatically.
34 Copper-catalysed azide alkyne cycloaddition reactions are chemoselective, fast and form only
35 one stereoisomer, with an irreversible linkage, under ambient conditions.¹⁹ The mild
36 conditions of this reaction have previously been applied to modify oligonucleotides,²⁰⁻²¹ to
37 functionalise nanoparticles with enzymes,²² and in fluorescent-labeling of cellular systems.²³

38 A series of modified oligonucleotides as precursors for OPC formation under click chemistry
39 conditions were synthesised. The alkyne could be added to either the peptide or the
40 oligonucleotide as could the azide group, and both scenarios were examined. To produce a 5'-
41 alkynyl modified oligonucleotide, 5-hexyn-1-ol was phosphitylated and incorporated into the
42 5'-end of the DNA sequence via solid phase synthesis. Conversely, a direct phosphoramidite
43 derivative of the azido function is not possible due to the reactivity of this group with
44 phosphines, i.e. via the Staudinger reaction. To overcome this and to produce an azido-
45 modified oligonucleotide, succinimidyl azidovalerate was synthesised and reacted with an
46 amino-modified solid support (Scheme 1). The Fmoc protecting group was removed using a
47 piperidine solution and the free amine was reacted with the activated ester before being used
48 with standard phosphoramidite chemistry to yield a 3'-azido-modified oligonucleotide.

49 In a similar approach, a 5'-azido-modified oligonucleotide was produced using a two-step
50 process: 5'-monomethoxytrityl (MMT) aminomodifier phosphoramidite was used to modify
51 the 5'-end of the oligonucleotide with a protected amine group. Removal of the MMT
52 protecting group allowed the free amine to react with succinimidyl azidovalerate to generate
53 a 5'-azido-modified oligonucleotide. The modified sequences were cleaved, purified and
54 characterized by MALDI-TOF mass spectrometry (Table 1).

55 Azide-modified Tat peptide was synthesised by reaction of the N-terminus of the peptide
56 with succinimidyl azidovalerate. Propiolic acid was coupled to the N-terminus of Tat peptide
57 to form an amide bond which gave the alkyne-modified peptide. Conjugation of the 5'-
58 alkyne-modified oligonucleotide with the azido-modified Tat peptide derivative
59 (YGRKKRRQRRR) and the 5'- and 3'-azido-modified oligonucleotides with alkyne-modified
60 Tat peptide was carried out using the reaction conditions as recommended by Kolcálka *et*
61 *al.*²⁴ This included tris(benzyltriazolylmethyl)amine (TBTA), an additional ligand which has
62 been shown to stabilise Cu(I) and accelerate the reaction (Scheme 2).²⁵ An aliquot of

63 formamide was added to ensure the covalent attachment of the biomolecules and prevent
64 them coming together electrostatically.¹⁷ Each solution was agitated at room temperature
65 overnight.

66 Ion-exchange HPLC analysis of the reaction between the 3'-azido-modified
67 oligonucleotide and the alkyne-modified peptide showed the formation of a new peak
68 with a shorter retention time than that of the unconjugated oligonucleotide (Figure 1).

69
70 The OPC is overall less negatively charged in comparison to the unconjugated
71 oligonucleotide as the ionic charges are negated due to the positively charged peptide.
72 The peak appearing at approximately 11 minutes, thought to be the OPC product, was
73 collected, dialysed to remove remaining formamide and further purified using ZipTip™
74 C₁₈ pipette tips. Formation of the OPC was confirmed by MALDI-TOF mass
75 spectrometry in positive mode (Table 1). Based on peak ratios, the conjugate was formed
76 in 56% yield.

77 All conjugation reactions described were carried out under argon atmospheres to prevent
78 breakdown of the copper catalyst. It was subsequently found, however, that this made no
79 difference to the outcome of the reactions. The arginine side chain is known to stabilise
80 Cu(I) which may prevent the anticipated oligonucleotide degradation negating the need
81 for an inert atmosphere.²⁶

82 No OPC peak was observed for the synthesis of the 5'-azido-modified or 5'-alkyne-modified
83 oligonucleotide-Tat peptide conjugates. It is not fully understood why the reaction between
84 5'-azido-modified oligonucleotide and alkyne-modified Tat peptide did not proceed, however
85 successful formation of oligonucleotide-Tat peptide conjugates may require an activated
86 alkyne which was present during the formation of OPC 1.²⁷ The amino acid side chains of the
87 peptide can have a significant effect on the reaction outcome and underlines the difficulty in
88 using biologically relevant peptides such as Tat.

89 In conclusion, a series of modified oligonucleotides as precursors for CuAAC synthesis of
90 OPCs were generated, however, OPC formation was only observed upon reaction with 3'-
91 azido-modified oligonucleotide and alkyne-modified Tat peptide. This is the first report of
92 the preparation of an OPC *via* CuAAC using Tat. The reaction proceeds under aerobic
93 conditions, at room temperature, in water to reportedly form one stereoisomer.^{19,28} These are
94 attractive properties in the development of biological tools for diagnostics and therapeutics.

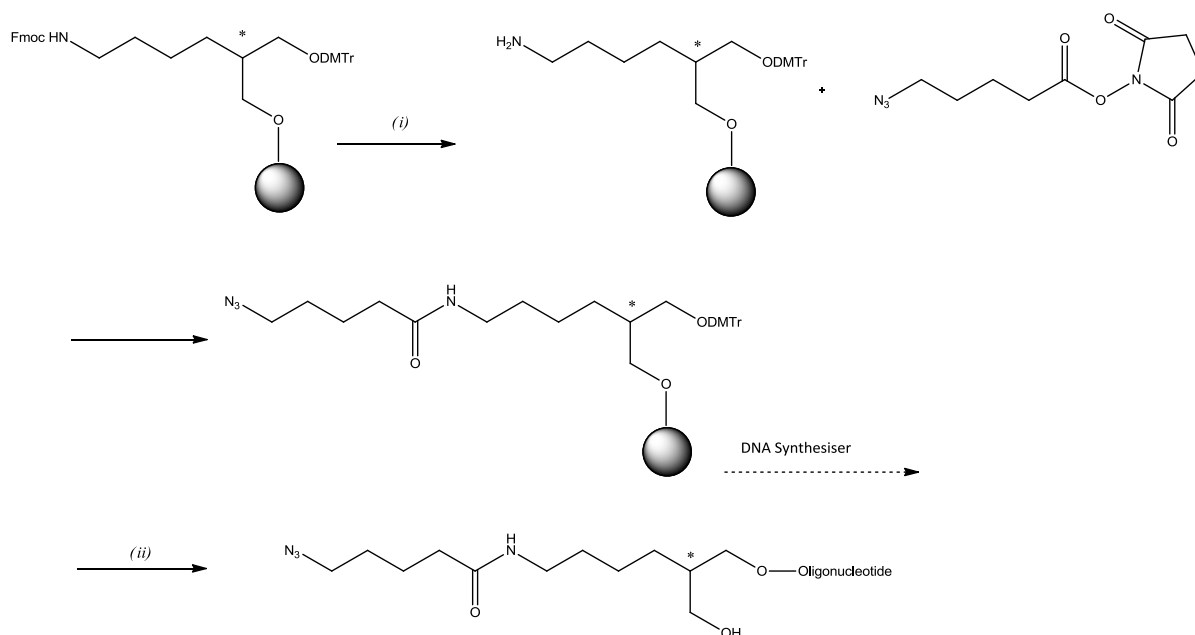
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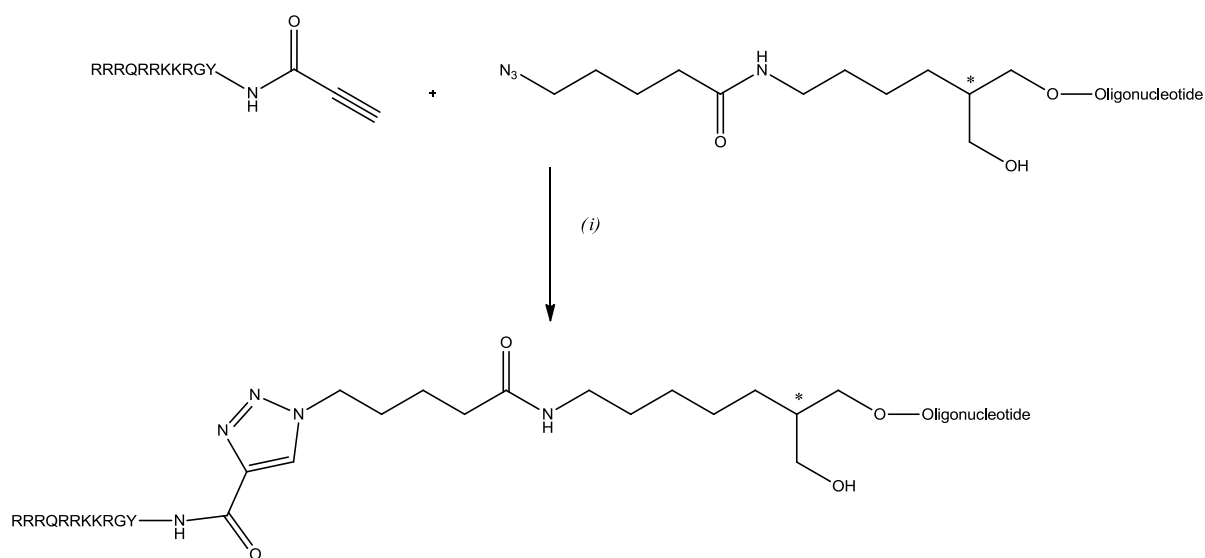
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138 **Scheme 1.** (i) 20% piperidine in MeCN; (ii) concentrated NH_4OH . *Indicates a chiral centre.

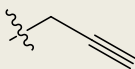


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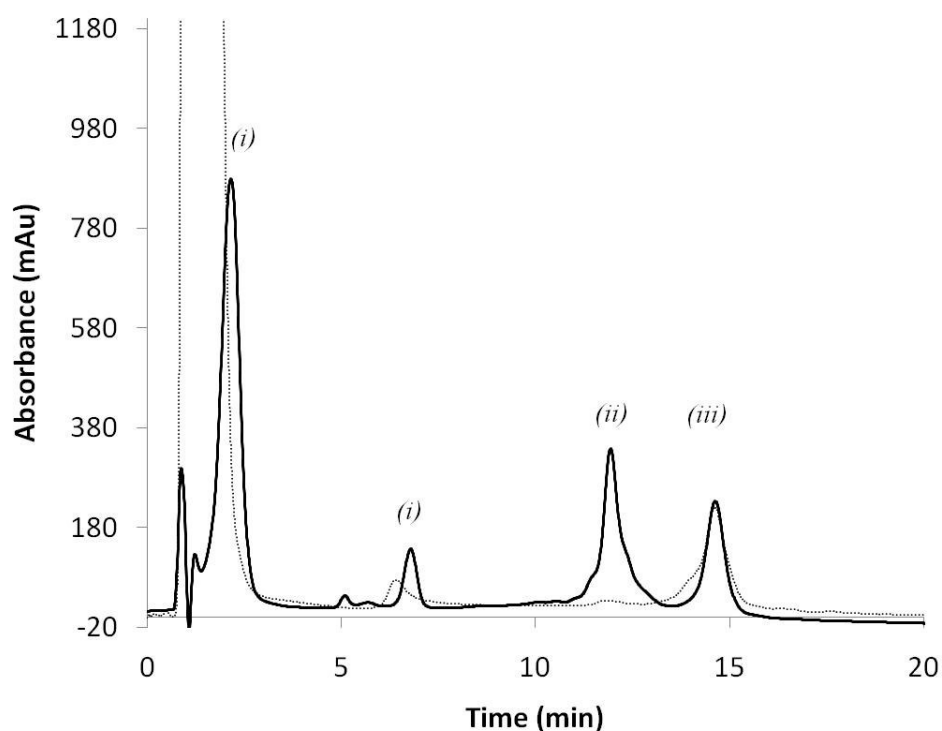
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141 **Scheme 2.** (i) TBTA, sodium ascorbate, CuSO_4 , formamide, phosphate buffer, 56 %.

142

	Calc'd m/z	Found m/z
X= 	5643.4	5642.2
Y= 	5785.3	5782.9
X= 	5740.1	5739.4
1	7396.0	7399.2

143 **Table 1.** MALDI-TOF mass spectrometric characterisation of modified oligonucleotides,
144 5'-X-GTT TTC CCA GTC ACG ACG-Y-3' and oligonucleotide-Tat peptide conjugate 1.



145

146 **Figure 1** Ion-exchange HPLC traces at 260 nm of 3'-azido-modified oligonucleotide-
147 Tat OPC (solid line) and control (dashed line): (i) unreacted catalyst mixture, (ii) OPC, (iii)
148 unreacted oligonucleotide. The control contained all the (TBTA, CuSO₄, sodium ascorbate,
149 3'-azido-modified oligonucleotide, formamide, phosphate buffer) but not the Tat peptide.