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

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

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

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

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

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

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

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

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

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

The OPC is overall less negatively charged in comparison to the unconjugated oligonucleotide as the ionic charges are negated due to the positively charged peptide. The peak appearing at approximately 11 minutes, thought to be the OPC product, was collected, dialysed to remove remaining formamide and further purified using ZipTip™ C_{18} pipette tips. Formation of the OPC was confirmed by MALDI-TOF mass spectrometry in positive mode (Table 1). Based on peak ratios, the conjugate was formed in 56% yield.

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

No OPC peak was observed for the synthesis of the 5′-azido-modified or 5′-alkyne-modified oligonucleotide-Tat peptide conjugates. It is not fully understood why the reaction between 5′-azido-modified oligonucleotide and alkyne-modified Tat peptide did not proceed, however successful formation of oligonucleotide-Tat peptide conjugates may require an activated alkyne which was present during the formation of OPC **1**.[27] The amino acid side chains of the peptide can have a significant effect on the reaction outcome and underlines the difficulty in using biologically relevant peptides such as Tat.

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

Acknowledgements

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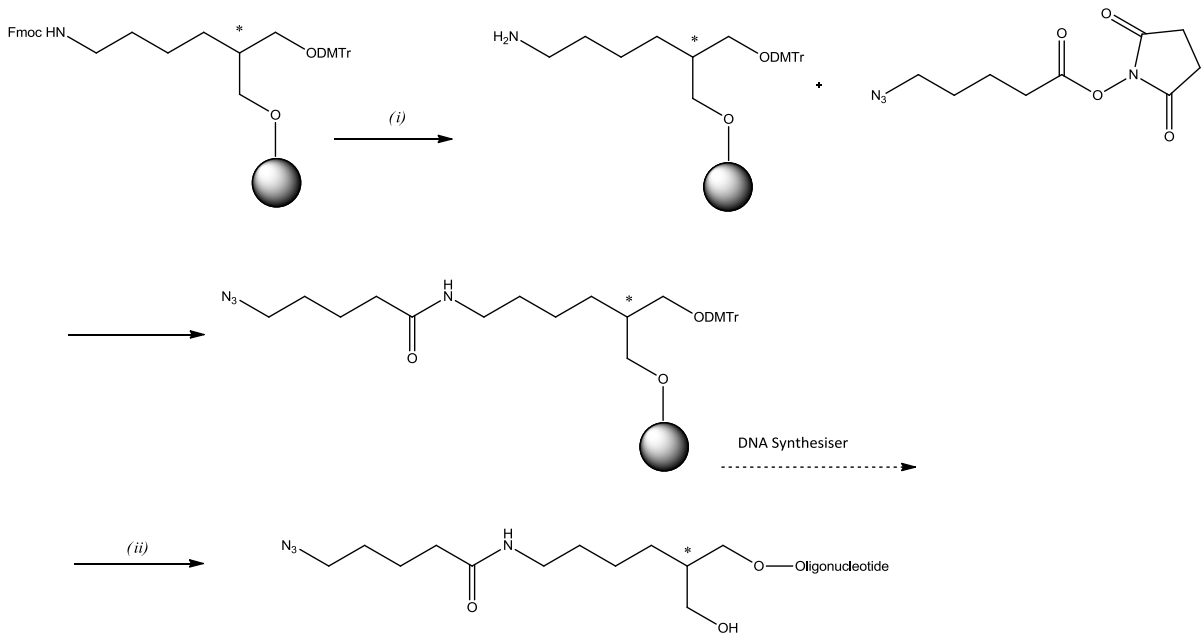

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138 **Scheme 1.** (i) 20% piperidine in MeCN; (ii) concentrated NH_4OH. *Indicates a chiral centre.

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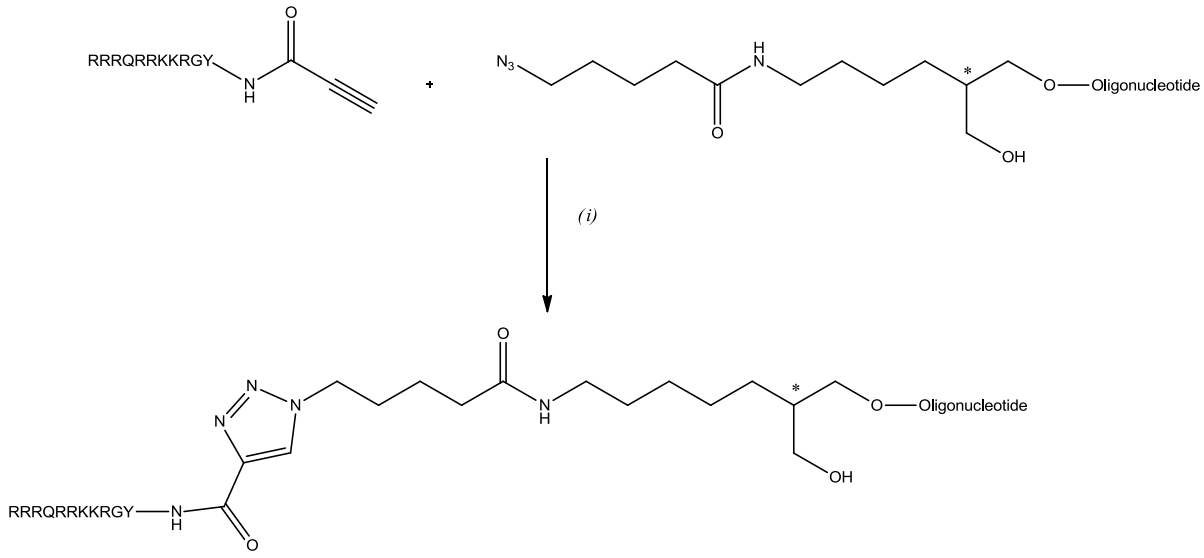

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141 **Scheme 2.** (i) TBTA, sodium ascorbate, $CuSO_4$, formamide, phosphate buffer, 56 %.

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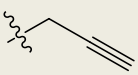
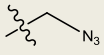
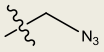
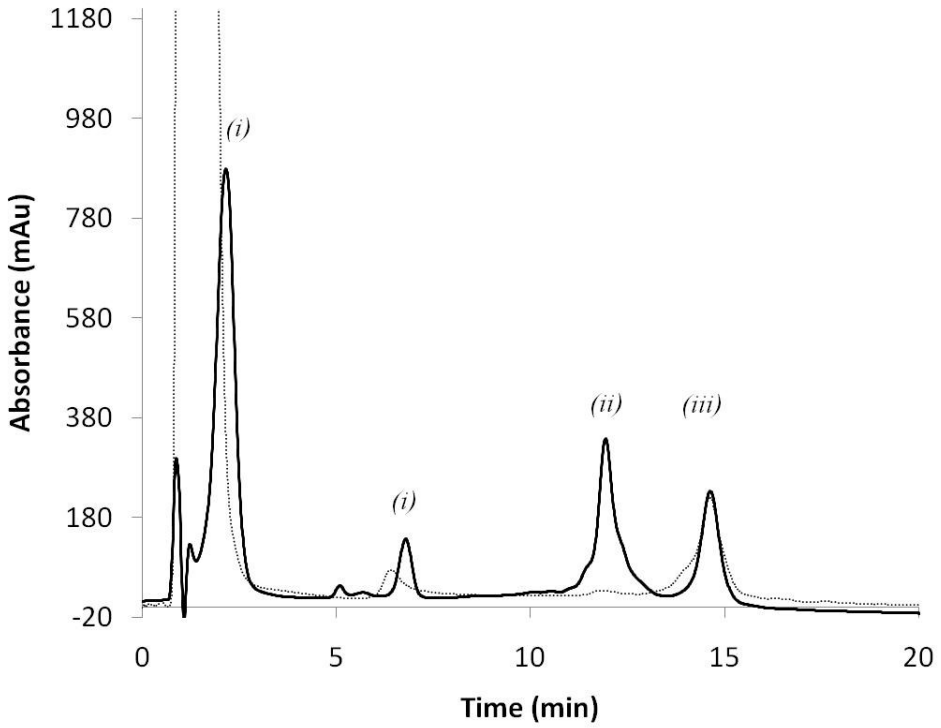
	Calc'd *m/z*	Found *m/z*
X= 	5643.4	5642.2
Y = 	5785.3	5782.9
X = 	5740.1	5739.4
1	7396.0	7399.2

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, $CuSO_4$, sodium ascorbate,
149 3'-azido-modified oligonucleotide, formamide, phosphate buffer) but not the Tat peptide.