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.

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Detection and treatment of a 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 the oligonucleotides.[2] Attachment of cell-penetrating peptides (CPPs) to oligonucleotides is well documented and has been found to facilitate transfection and enhance resistance to the degradation of nucleic sequences.[3–9] The conflicting chemistries of peptide and oligonucleotide synthesis make in-line conjugation challenging. Total solid-phase synthesis overcomes these problems, however, the method is not very flexible.[10] Synthesising the two biomolecules and linking them in solution (fragment conjugation) avoid these problems but can be labour intensive, can be 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 from reaction mixtures.[16,17] While Gogoi et al. have produced oligonucleotide–peptide conjugates (OPCs) using click chemistry,[18] we now 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 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-labelling 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 the 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, that is, 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 piperidine and the free amine was reacted with the activated ester before being used with standard phosphoramidite chemistry to yield a 3′-azido-modified oligonucleotide.

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

	Calcd 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

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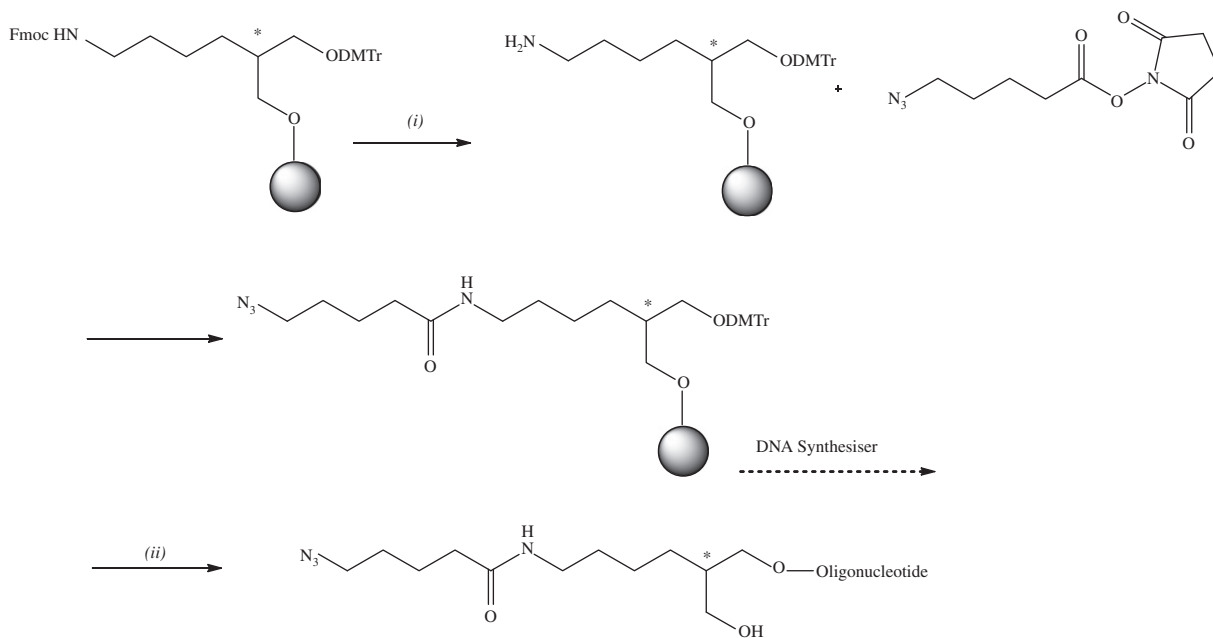
Scheme 1. Reagents: (i) 20% piperidine in MeCN; (ii) concentrated NH₄OH. *indicates a chiral centre.

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 a 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 the 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 (Fig. 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 min, thought to be the OPC product, was collected, dialysed to remove the remaining formamide and further purified using ZipTip™ C₁₈ 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 to prevent the breakdown of the copper catalyst. It was subse-
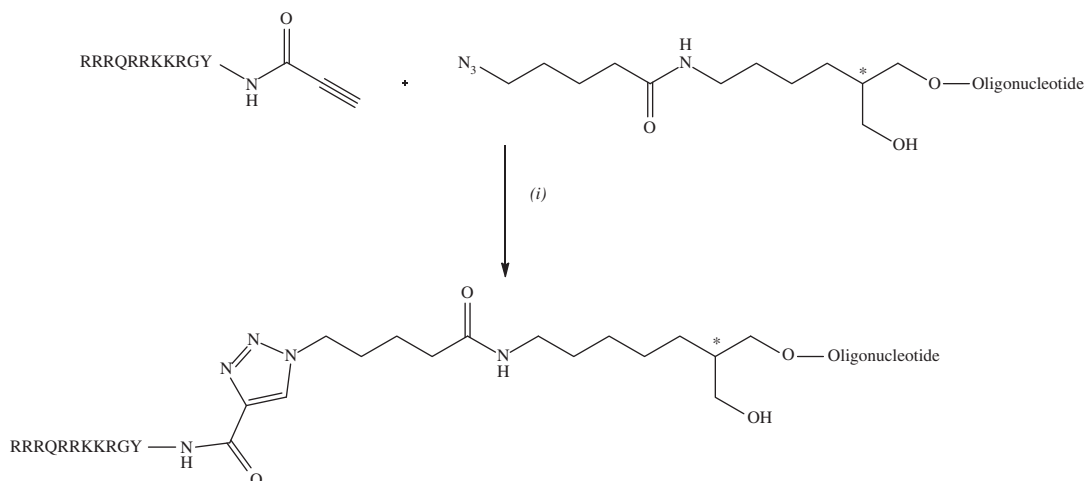
Scheme 2. Reagents: (i) TBTA, sodium ascorbate, CuSO₄, formamide, phosphate buffer, 56%.
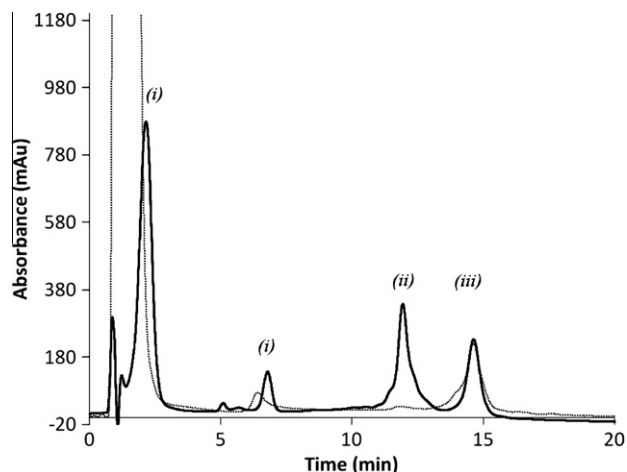
Figure 1. Ion-exchange HPLC traces at 260 nm of 3′-azido-modified oligonucleotide–Tat OPC (solid line) and a control (dashed line): (i) unreacted catalyst mixture, (ii) OPC, (iii) unreacted oligonucleotide. The control contained TBTA, CuSO$_4$, sodium ascorbate, 3′-azido-modified oligonucleotide, formamide, phosphate buffer, but not the Tat peptide.

quently 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 as to why the reaction between 5′-azido-modified oligonucleotide and alkyne-modified Tat peptide did not proceed, however, the 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 this 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 a 3′-azido-modified oligonucleotide and an 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, and at room temperature, in water.[19,28] These are attractive properties in the development of biological tools for diagnostics and therapeutics.

Supplementary data

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