

Solid phase click ligation for the synthesis of very long oligonucleotides†

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Oligonucleotides have been ligated efficiently on solid-phase using CuAAC and SPAAC chemistry to produce up to 186-mer triazole linked DNA products. Multiple sequential ligation reactions can be carried out by using a masked azide approach. This work suggests a novel modular approach to the synthesis of large complex oligonucleotide analogues.

The standard approach to the chemical synthesis of DNA is the highly efficient solid-phase phosphoramidite method.¹ It can be used to assemble oligonucleotides over 100 bases in length, beyond which limitations in the chemistry make it difficult to produce pure DNA. An approach to the synthesis of larger DNA strands is to combine solid-phase oligonucleotide synthesis with chemical ligation, for example by using cyanogen bromide as a coupling agent.^{2,3} However, it remains challenging to achieve clean and efficient chemical ligation of oligonucleotides. An alternative strategy is to design a chemical linkage that mimics the natural phosphodiester and which can be formed efficiently and selectively. This has been achieved through a click chemistry approach⁴ in which the Cu^I-catalysed [3+2] azide-alkyne cycloaddition (CuAAC) reaction^{5,6} is used to synthesise DNA containing biocompatible artificial linkages (Fig. 1a). An alternative copper-free DNA ligation method has recently been developed for applications in which it is not necessary to copy or amplify the sequence.^{7,8} This strategy takes advantage of the strain-promoted alkyne azide cycloaddition (SPAAC) reaction⁹ between azide and cyclooctyne-modified oligonucleotides (Fig. 1b). Both reactions are carried out in aqueous buffer in the presence of a complementary oligonucleotide template (splint) to hold the participating oligonucleotides in place. We now report a solid-phase strategy for ligating oligonucleotides using the CuAAC and SPAAC reactions. This approach has several desirable features; it is simple to carry out, the reaction can be forced to completion by adding an

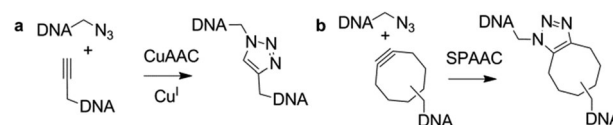


Fig. 1 CuAAC and SPAAC click reactions for DNA ligation.

excess of the solution-phase reactant, there is no requirement for a template oligonucleotide, and excess reagents can be conveniently removed and recovered.

The solid-phase CuAAC reaction has previously been used to synthesise short oligonucleotides with 5'-5'-linkages.¹⁰ In the present work it was investigated for the synthesis of long oligonucleotides containing the biocompatible triazole linkage **tz1** (Scheme 1c) which has applications in gene synthesis.^{11,12} The requisite 5'-azide oligonucleotides (ON1, 2, 5) were prepared on solid support by incorporating 5'-iodo-dT phosphoramidite monomer in the final coupling step followed by displacement of iodine with azide¹³ (Scheme 1a). The corresponding 3'-alkyne oligonucleotides (ON3, ON7) were prepared using protected 3'-O-propargyl-5-methyl-deoxycytidine solid support,¹¹ after which they were cleaved from the resin, deprotected and purified by HPLC (Scheme 1b). The reaction between a 5'-azide labelled oligonucleotide on the solid support and a 3'-alkyne labelled oligonucleotide in solution (Scheme 1c) were conducted on a 2 nmol scale.

The resin-bound 17-mer azide oligonucleotide (Table 1) was made on cpg support of two different pore sizes; 1000 Å (ON1) and 3000 Å (ON2). It was reacted with 3 equivalents of 3'-alkyne-labelled 10-mer ON3 in aqueous CuSO₄, ascorbate, Cu^I-binding ligand (Scheme 1) and 50% v/v DMF. In both cases the reaction was complete within 7 h at 70 °C or 21 h at 40 °C (Table 2 and Fig. 2a) to give the triazole product (ON4) in high purity (~90%). No significant difference was observed between the reactions using the two different resin pore sizes, and 3000 Å resin was used for further conjugations involving long oligonucleotides.

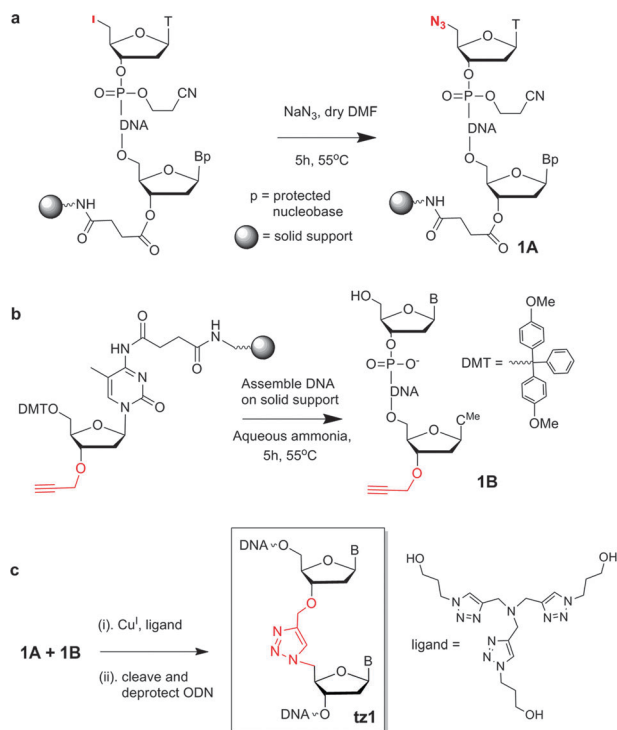
Next a much longer support-bound oligonucleotide (ON5, 61-mer) was conjugated to ON3 under similar conditions. The reaction was complete in 21 h at 55 °C and the 72-mer (ON6) was obtained in good yield after cleavage from the resin and deprotection (Fig. 2b).

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† Electronic supplementary information (ESI) available: Experimental details for oligonucleotide synthesis, purification and labelling, click reactions, PAGE gels, full HPLC and mass spectra of click ligation reactions, table of oligonucleotide sequences and mass spec characterisation. See DOI: 10.1039/c3cc42451k





Scheme 1 Synthesis of biocompatible triazole linked oligonucleotides on solid-phase.

Table 1 Oligonucleotides used in this study. For CuAAC reactions: (Scheme 1). X = 3'-propargyl 5-MedC, 5'-N₃ = 5'-azido, **tz1** = biocompatible triazole. For SPAAC reactions (Fig. 3): 5'-BCN is added using phosphoramidite **2**, 3'-BCN is made from **1** + **4**, 3'-N₃ is made from **3** + **4**. Structures of **tz2**, **tz3**, **tz4**, **1**, and **4** are in Fig. 3

Code	Sequence (5'–3')	Code	Sequence (5'–3')
ON1	5'-N ₃ -17mer-1000 Å resin	ON13	5'-BCN-60mer-3000 Å resin
ON2	5'-N ₃ -17mer-3000 Å resin	ON14	60mer-N ₃ 3'
ON3	10-mer-X	ON15	ON13-tz3-ON14
ON4	ON1/2-tz1-ON3	ON16	5'-I-60mer-N ₃ 3'
ON5	5'-N ₃ -61mer-3000 Å resin	ON17a	5'-I-ON16-tz3-ON13
ON6	ON3-tz1-ON5	ON17b	5'-N ₃ -ON16-tz3-ON13
ON7	59mer-X	ON18	ON17b-tz2-ON11
ON8	ON1-tz1-ON7	ON19	5'-I-25mer-N ₃ 3'
ON9	11mer-BCN3'	ON20a	5'-I-ON19-tz3-ON13
ON10	ON1-tz2-ON9	ON20b	5'-N ₃ -ON19-tz3-ON13
ON11	66mer-BCN3'	ON21	ON20b-tz2-ON11
ON12	ON1-tz2-ON11	ON22	ON5-tz4-ON13 (5'–5'-link)

Table 2 Solid-phase CuAAC reaction between 3'-alkyne ONs and support-bound 5'-azide ONs. Products characterised by MS (ESI⁺). Full conditions in ESI

Azide ON	Alkyne ON	Alkyne eq.	Time °C	Product hours	Product code	Length	MS calc.	MS found
ON1	ON3	3	70	7	ON4	28	8403	8402
ON1	ON3	3	40	21	ON4	28	8403	8402
ON2	ON3	3	70	7	ON4	28	8403	8402
ON2	ON3	3	40	21	ON4	28	8403	8402
ON5	ON3	3	55	21	ON6	72	22 232	22 232
ON1	ON7	10	70	16	ON8	77	23 579	23 579

The reaction at 40 °C did not quite proceed to completion, even after 21 h. Similar results were obtained when the reaction was carried out using the shorter azide oligonucleotide on solid-phase (ON1) and the long 3'-alkyne oligonucleotide (59-mer ON7) to give ON8. Further

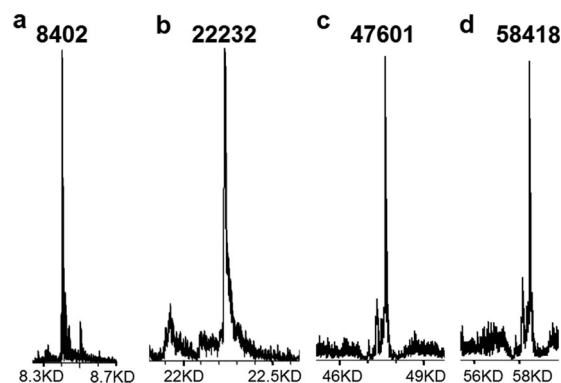


Fig. 2 ESI⁺ MS of click-ligated oligonucleotides. (a) ON4, (b) ON6, (c) ON21 and (d) ON18. Full HPLC chromatograms and mass spectra are shown in ESI⁺.

experiments confirmed that the optimum reaction conditions were 10 eq. of the alkyne oligonucleotide at 70 °C for 16 h. Higher temperatures and longer times led to some Cu^I-catalysed oligonucleotide degradation despite the presence of the tris-hydroxypropyltriazolide ligand¹⁴ (Scheme 1c).

For applications in which there is no requirement for polymerase enzymes to read through the triazole linkage the SPAAC reaction is an excellent alternative; it is extremely fast and does not require Cu^I catalysis. The highly active symmetric bicycle [6.1.0] non-4-yne (BCN)¹⁵ was used as the strained alkyne component to avoid diastereomers or regioisomers of the triazole products. It has recently been used for solution-phase oligonucleotide crosslinking and fluorescent labelling.⁸ Dibenzocyclooctyne (DIBO) was also found to be a suitable alkyne in solid-phase SPAAC ligation reactions, but gave the expected regio/stereoisomers (ESI⁺).

BCN was attached post-synthetically to several 3'-amino-C7-labeled oligonucleotides **4** using BCN NHS carbonate **1** (Fig. 3a). Support-bound azide ON1 was reacted with ON9 in a 1:1 ratio for 21 h at 40 °C, to give quantitative conversion to the triazole ligation product ON10. A conjugation reaction was then conducted between 3'-BCN 66-mer (ON11) and 5'-azide oligonucleotide on resin (ON1). After 21 h at 40 °C the reaction proceeded to completion to give ON12. Next a solution-phase 3'-azide oligonucleotide was reacted with a support-bound 5'-BCN oligonucleotide. The required 3'-azide oligonucleotide (ON14) was synthesized by coupling azidohecanoic acid NHS ester **3** (ref. 16) to the 3'-amino-C7 oligonucleotide precursor **4** (Fig. 3a), and to prepare the support-bound 60-mer alkyne oligonucleotide (ON13), BCN phosphoramidite (**2**, Fig. 3a) was added to the 5'-terminus during solid-phase synthesis. This was reacted with 60-mer ON14 in aqueous solution (1.5 eq.) for 21 h at 40 °C, resulting in the efficient formation of 120-mer ON15 containing triazole backbone **tz3** (Fig. 3c and Table 3).

The success of the solid-phase SPAAC reaction to ligate two long oligonucleotides suggests that multiple SPAAC ligation reactions might be possible (Fig. 4). This requires the use of solution-phase oligonucleotides containing azide at each end. To control the regioselectivity of this reaction and prevent the formation of a 5'–5' linkage it was necessary to mask the 5'-azide function. To achieve this, ON16 and ON19 were synthesized with 5'-iodo-dT and 3'-azide (derived from **3** + **4**, Fig. 3a) using fast-deprotection A, G and C monomers (ESI⁺). The oligonucleotides were cleaved from the resin and deprotected in ammonia at room temperature for 5 h to



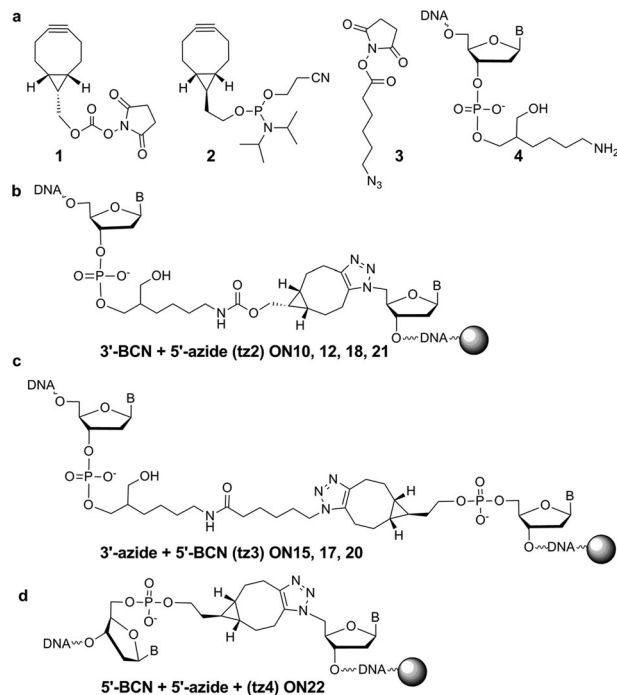


Fig. 3 Reagents and products of SPAAC ligation reactions. BCN reagents **1** and **2** from Berry Associates, for azide NHS ester **3** see ref. 16.

Table 3 Solid-phase SPAAC reactions in 10% pyridine in water, 21 h reaction time at 40 °C. Products characterised by MS (ESI[−]). Full conditions in ESI

Azide ON	Alkyne ON	Solution ON eq.	Product code	Length	MS calc.	MS found
ON1	ON9	1.0	ON10	28	8735	8736
ON1	ON11	1.0	ON12	83	25 845	25 846
ON14	ON13	1.5	ON15	120	37 617	37 618
ON16	ON13	3.0	ON17b	120	37 615	37 615
ON17b	ON11	3.0	ON18	186	58 417	58 418
ON19	ON13	3.0	ON20b	85	26 798	26 798
ON20b	ON11	3.0	ON21	151	47 600	47 601
ON5	ON13	3.0	ON22	121	37 641	37 642

preserve the integrity of the 5'-iodo group. They were then individually conjugated in a 3 : 1 ratio to the support-bound 60-mer ON13 to give triazole products ON17a and ON20a attached to the solid support. The 5'-iodo group was then displaced on solid phase by azide using DMF-NaN₃ (Scheme 1a) to give ON17b and ON20b. ON11 was then reacted with these click-linked oligonucleotides on solid support. Cleavage and deprotection gave the full length 151-mer and 186-mer products ON21 and ON18 (Fig. 2c, d and 4). Finally the solid-phase synthesis of 5'-5'-linked oligonucleotides was demonstrated by ligation of ON5 on solid support to ON13 in solution to give 121-mer ON22. It is noteworthy that in solid-phase SPAAC ligation reactions it is possible to recover the unreacted solution-phase oligonucleotide for re-use simply by lyophilisation.

Conditions for the solid-phase ligation of oligonucleotides by the CuAAC and SPAAC reactions have been developed which allow the efficient assembly of oligonucleotides up to 186 bases in length. Multiple sequential ligation reactions can be carried out with no requirement for a template oligonucleotide. This solid-phase method

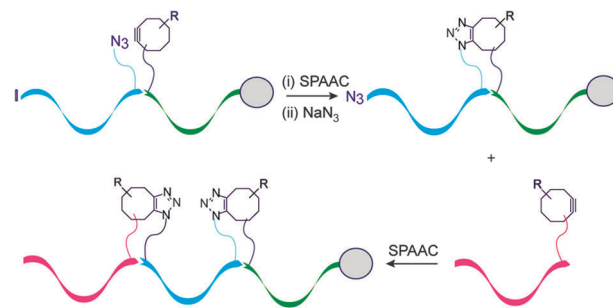


Fig. 4 Double sequential SPAAC ligation reactions using 5'-iodo as a masked 5'-azide to synthesise 186-mer ON18 and 151-mer ON21.

should be valuable for the synthesis of oligonucleotides conjugated to DNA analogues such as PNA. Many other applications can be envisaged, e.g. the synthesis of libraries of oligonucleotides which all have different 3'-sections (PCR primer or probe elements) but the same 5'-section, e.g. regions that contains several expensive fluorescent dyes or other complex modifications. The individual 3'-parts could be made on a small scale on solid-phase with a 5'-BCN attachment, and the 5'-part could be made on a large scale with a 3'-azide. The purified 3'-azide oligonucleotide could then be clicked to all the different resin bound 3'-components to give a library of oligonucleotides. If this is intended for use as a cocktail of probes in applications such as fluorescence *in situ* hybridization (FISH), all the resin-bound 3'-components can be mixed and ligated to the 5'-component in a single SPAAC reaction. We envisage that the methodology presented here will also be invaluable for the synthesis of very long modified oligonucleotides with complex 3-D architectures for use in various nanotechnology applications.

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