View Online / Journal Homepage / Table of Contents for this issue
Dynamic Article Links

ChemComm

Cite this: *Chem. Commun.*, 2011, **47**, 6257–6259

www.rsc.org/chemcomm

COMMUNICATION

Fast copper-free click DNA ligation by the ring-strain promoted alkyne-azide cycloaddition reaction†

Montserrat Shelbourne,[a] Xiong Chen,[a] Tom Brown‡*[a] and Afaf H. El-Sagheer‡*[ab]

Received 8th February 2011, Accepted 12th April 2011
DOI: 10.1039/c1cc10743g

Downloaded by North Carolina State University on 27 August 2012
Published on 06 May 2011 on http://pubs.rsc.org | doi:10.1039/C1CC10743G

Templated DNA strand ligation by the ring-strain promoted alkyne–azide [3 + 2] cycloaddition reaction is very fast; with dibenzocyclooctyne, the reaction is essentially complete in 1 min. It is inhibited by the presence of a single mismatched base pair suggesting applications in genetic analysis.

The CuAAC 'click reaction' (Cu^{I}-catalysed [3 + 2] alkyne azide cycloaddition) has been used in a vast range of applications due to its high efficiency and orthogonality with almost all other functional groups and solvents.[1] It has found favour in the nucleic acids field[2] as a method of joining together single strands of DNA, cross-linking complementary strands,[2,3] cyclising single and double strands,[4,5] labelling oligonucleotides with reporter groups,[2,6–9] attaching DNA to surfaces,[2] producing analogues of DNA with modified nucleobases[2,7,10,11] and backbones,[2,12–14] synthesising large chemically modified RNA constructs[15] and creating biochemically active PCR templates.[12] However, Cu^{I} has undesirable cytotoxicity even at low concentrations[16] so it is not fully compatible with *in vivo* applications. Unfortunately, the uncatalysed DNA-templated AAC reaction with terminal alkynes is exceedingly slow in comparison[17] unless highly activated alkynes are used, and these are unstable in water. Clearly, it would be advantageous to have the option to carry out click chemistry on DNA in the absence of metal ion catalysis. For carbohydrates this problem has been elegantly solved[16,18–22] by the introduction of the ring strain-promoted azide–alkyne [3 + 2] cycloaddition reaction (SPAAC).[23,24] This involves the reaction between an azide and a strained alkyne such as a cyclooctyne. Here, we have adopted this approach for nucleic acids and describe the use of cyclooctyne derivatives in Cu^{I}-free templated DNA ligation (Fig. 1).

The aim of this study was to discover alkynes that would react quickly with azides at low concentration under DNA-templated conditions, but remain unreactive in a non-templated mode. This should be achievable, as DNA templation accelerates reaction rates by several orders of magnitude.[17] To achieve this objective we have synthesised two activated cyclooctyne derivatives (Fig. 2) for conjugation to amino-functionalised oligonucleotides; the non-substituted cyclooctyne (NSCO, **1**) and the previously reported dibenzocyclooctynol (DIBO, **2**).[25] We anticipated that alkyne **2** would be more reactive towards azides than **1** because the aromatic rings are expected to impose additional ring strain and electron withdrawing properties.[25] For the method to be suitable for the controlled simultaneous ligation of several oligonucleotides it is important that the ligation reaction occurs only in the presence of a complementary DNA template so that only the desired products are formed. Therefore it was necessary to compare reactivity under both templated and non-templated conditions.

Initially both alkynes were attached post-synthetically to the 5′-end of an aminohexyl-labelled oligonucleotide (ODN-1, 2, Table 1). The NHS ester of 6-azidohexanoic acid was added to a 3′-amino alkyl labelled oligonucleotide to provide the azide oligonucleotide (ODN-3) which has a fluorescein dye at the 5′-end to allow visualisation at low concentrations. HPLC purification was carried out on all oligonucleotides and the products were characterised by mass spectrometry (ESI). Templated and non-templated ligation reactions between azide ODN-3 and alkyne ODN-1 and ODN-2 were carried out in the absence of Cu^{I}.

Of the two cyclooctynes tested, DIBO (**2**) was much faster in templated ligation (Fig. 3). Reactions with this alkyne proceeded cleanly and were essentially complete within 1 min at 2 μM DNA concentration. NSCO (**1**) also reacted cleanly but required more than 30 min for complete reaction. In both cases the non-templated reactions gave little or no product under otherwise identical conditions (Fig. 3). Importantly, introduction of a single mismatch base pair between template ODN-11 and DIBO-labelled ODN-1 was sufficient to inhibit the ligation reaction (Fig. 3 lanes 17, 18), pointing to future applications in genetic analysis.

Interestingly the addition of Cu^{I} strongly inhibited the SPAAC reaction (Fig. 4a lanes 4, 5, 8, 9 and ESI†). There are two possibilities here: either the alkyne is deactivated on complexation to Cu^{I}, or degrades when exposed to the metal ion. Treatment of the cyclooctyne ODNs with Cu^{I} indicated that no degradation occurred (analysis by mass spec and gel

[a] *School of Chemistry, University of Southampton, Highfield, Southampton, SO17 1BJ, UK. E-mail: ahes@soton.ac.uk, tb2@soton.ac.uk*
[b] *Chemistry Branch, Dept. of Science and Mathematics, Faculty of Petroleum and Mining Engineering, Suez Canal University, Suez 43721, Egypt*

† Electronic supplementary information (ESI) available: Experimental details, gel electrophoresis, UV melting studies, oligonucleotide mass spectral data. See DOI: 10.1039/c1cc10743g
‡ AHE-S and TB are joint main authors.

View Online

Downloaded by North Carolina State University on 27 August 2012
Published on 06 May 2011 on http://pubs.rsc.org | doi:10.1039/C1CC10743G

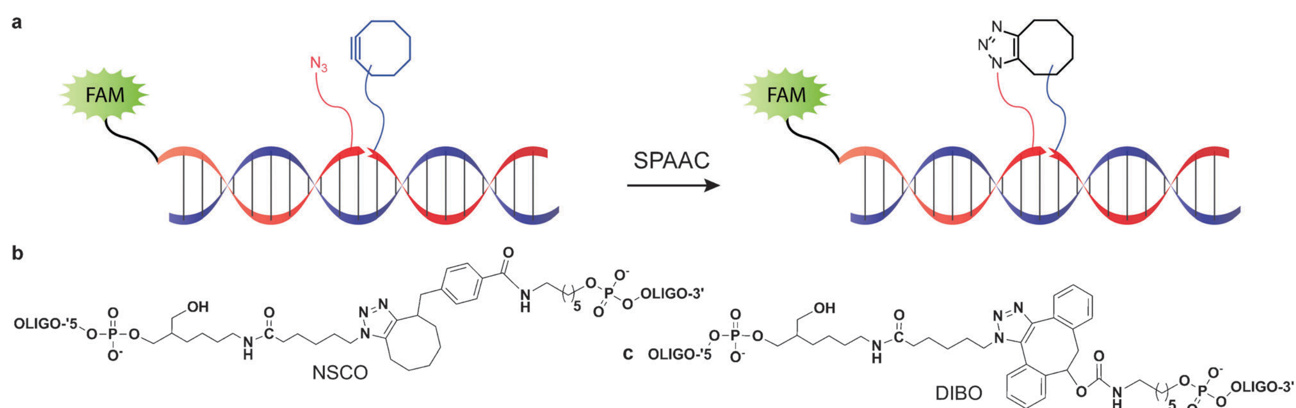

Fig. 1 SPAAC click DNA ligation between azide-labelled and cyclooctyne-labelled oligonucleotides. (a) schematic of reaction (FAM = 6-carboxyfluorescein). (b, c) chemical structures of NSCO and DIBO alkynes respectively at the ligation point.

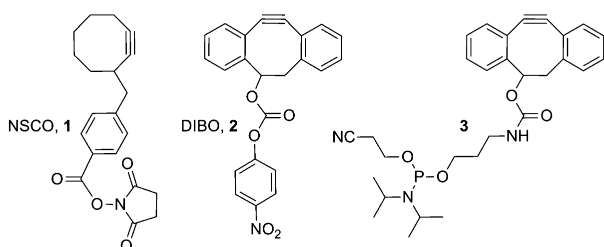

Fig. 2 (1, 2) Activated cyclooctynes for labelling amino-modified oligonucleotides. (3) DIBO phosphoramidite for insertion during solid-phase oligonucleotide synthesis.

Table 1 Oligonucleotide sequences. F = fluorescein, z = amino C7 labelled with 6-azidohexanoic acid, ^{C}K = aminohexyl labelled with NSCO (1), ^{DIBO}K = aminohexyl labelled with DIBO (2), $^{DIBO}K_1$ = dibenzocyclooctynyl derived from phosphoramidite (3). X, Y and Z = ligated triazole products derived from ODN-3 with ODN-1, ODN-2 and ODN-4 respectively. Fz^{DIBO} = 6-fluoresceinamidopropylazide + DIBO

Code	Oligonucleotide sequences
ODN-1	^{DIBO}K-GCGATCAATCAGACG
ODN-2	^{C}K-GCGATCAATCAGACG
ODN-3	F-CTTTCCTCCACTGTTGCz
ODN-4	$^{DIBO}K_1$-GCGATCAATCAGACG
ODN-5	TTTATTGATCGCGCAACAGTGTTT
ODN-6	F-CTTTCCTCCACTGTTGC<u>X</u>GCGATCAATCAGACG
ODN-7	F-CTTTCCTCCACTGTTGC<u>Y</u>GCGATCAATCAGACG
ODN-8	F-CTTTCCTCCACTGTTGC<u>Z</u>GCGATCAATCAGACG
ODN-9	Fz$^{DIBO}K_1$-GCGATCAATCAGACG
ODN-10	CTTTCCTCCACTGTTGCGCGATCAATCAGACG
ODN-11	TTTATTCATCGCGCAACAGTGTTT

electrophoresis). To test the stability of the putative complex with cuprous ion, the alkyne oligonucleotides were treated with Cu^I then all small molecules were removed by sephadex gel-filtration before carrying out the SPAAC reaction. This process did not restore the reactivity of the alkyne suggesting that the alkyne-Cu^I complex survived gel-filtration (Fig. 4a). It is known that Cu^I readily forms a complex with cyclooctyne[26] (Fig. 4b) and we assume that such a complex prevents the SPAAC reaction from occurring. The complex was not sufficiently stable to be observed by mass spectrometry of the oligonucleotides. Ethylenediamine tetraacetic acid (EDTA) (100 equ.) partially restored the reactivity of the cyclooctyne oligonucleotide (ESI†).

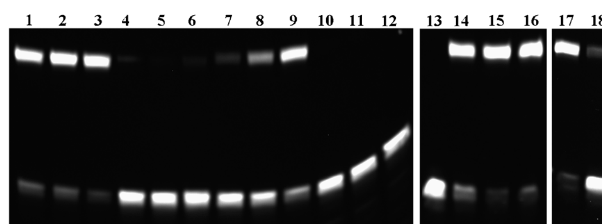

Fig. 3 SPAAC templated and non-templated reactions comparing DIBO and NSCO alkyne oligonucleotides. Lanes 1–3: templated reactions using DIBO ODN-1; 0 min, 5 min, 30 min, RT, lanes 4–6: non-templated reactions using DIBO ODN-1; 0 min, 5 min, 30 min, RT, lanes 7–9: templated reactions using NSCO ODN-2; 0 min, 5 min, 30 min, RT, lanes 10–12: non-templated reactions using NSCO ODN-2; 0 min, 5 min, 30 min, RT, lane 13: azide ODN-3, lanes 14–16: templated reactions using DIBO ODN-1; 1 min, 3 min, 5 min, RT, lanes 17 and 18 discrimination between fully matched and mismatched templated reactions using DIBO ODN-1 and templates ODN-5, (fully matched, lane 17) and ODN-11 (single base pair mismatch, lane 18); 5 min, 45 °C. All reactions performed at 2 μM oligonucleotide conc. in 0.2 M aq. NaCl for the specified time then mixed with formamide and loaded directly onto a 20% polyacrylamide gel.

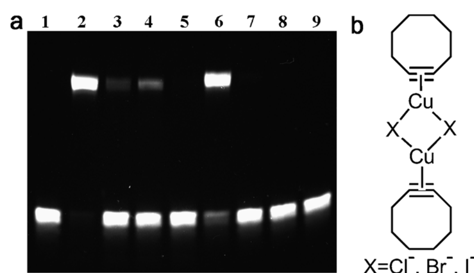

Fig. 4 (a) SPAAC templated and non-templated reactions using alkyne oligonucleotides pre-treated with Cu^I. Lane 1: azide ODN-3, lanes 2 and 3: control, templated and non-templated SPAAC reactions using the DIBO alkyne oligonucleotide (ODN-1), lanes 4 and 5: templated and non-templated reactions using DIBO oligonucleotide (ODN-1) pre-treated with Cu^I, lanes 6 and 7: control; templated and non-templated SPAAC reactions using the NSCO alkyne oligonucleotide (ODN-2), lanes 8 and 9: templated and non-templated reactions using NSCO oligonucleotide (ODN-2) pre-treated with Cu^I. All reactions carried out at 2 μM DNA conc. in 0.2 M NaCl for 30 min at room temperature before loading directly onto a 20% polyacrylamide gel. (b) Cu(I)-cyclooctyne complex.[26]

 This journal is © The Royal Society of Chemistry 2011

View Online

Downloaded by North Carolina State University on 27 August 2012
Published on 06 May 2011 on http://pubs.rsc.org | doi:10.1039/C1CC10743G

Ultraviolet melting studies on duplexes containing the alkynes NSCO (**1**) and DIBO (**2**) showed a decrease of ~7 °C in melting temperature (T_m) compared with the unmodified duplex which had a T_m of 66.1 °C (ESI†). The similarity in T_m for both ligated oligonucleotides was expected as both triazole linkers are quite long. If the linker was short there might be a difference in stability between duplexes containing the two different alkynes as DIBO is more bulky, and might also participate in aromatic stacking interactions with the nucleobases.

It would be more convenient if the alkyne functionality could be added to oligonucleotides during solid-phase synthesis rather than post-synthetically. This would provide the necessary orthogonality to facilitate the synthesis of cyclooctyne oligonucleotides which also contain sensitive fluorescent dyes or other labels which have to be added as active esters after solid-phase synthesis. With this in mind, and to evaluate the effect of the length of cyclooctyne linker on click reactivity, we prepared the phosphoramidite derivative **3** of DIBO (Fig. 2 and ESI†). The cyclooctyne was completely stable to oligonucleotide synthesis and deprotection conditions, including heating in conc. aqueous ammonia for 5 h at 55 °C. Pure alkyne-labelled oligonucleotides were readily obtained and these gave efficient templated ligation with azide oligonucleotides (ESI†). UV melting of the duplex containing the ligated product (ODN-8) from phosphoramidite **3** (propylcarbamoyl linker) gave a similar T_m to that obtained from the ligated product (ODN-6) derived from the alkyne active ester (amidohexyl linker) (ESI†).

Oligonucleotide labelling with fluorophores is of major importance for DNA diagnostics, sequencing and related applications.[27] Some fluorophores can be incorporated into oligonucleotides during solid-phase synthesis but many are labile, and therefore unsuitable for direct insertion. In such cases an alternative strategy is to introduce fluorophores post-synthetically by the use of amine/NHS ester, thiol/iodoacetamide or thiol/maleimide chemistry. These methods have limitations, as the electrophiles rapidly decompose in aqueous media. In recent years click chemistry has become an important alternative; it provides the highest conjugation efficiency and uses very stable, robust alkynes and azides. Oligonucleotide labelling using the copper-free SPAAC reaction would constitute a significant advance and could in principle be carried out *in vivo*. To evaluate this chemistry, labelling of ODN-4 with 6-carboxyfluorescein azide (10 eq.) was performed at 37 °C for 1 h, resulting in complete conversion to labelled oligonucleotide ODN-9 as two isomeric fluorescent products (ESI†). While working on this manuscript two independent reports have appeared on the use of this methodology for adding tags to DNA.[28,29]

In summary, two cyclooctynes have been incorporated into oligonucleotides and used in SPAAC reactions. Templated DNA ligation was very fast and a single base pair mismatch was sufficient to strongly inhibit the reaction. It should be possible to use this approach for multiple simultaneous templated DNA ligation reactions if participating oligonucleotides are labelled with either two alkynes or two azides. The SPAAC reaction is orthogonal to amide bond formation and the Diels–Alder reaction on DNA[30] and, unlike the CuAAC reaction, does not require catalysis with toxic metals. Importantly the cyclooctyne and azide oligonucleotides are both stable in aqueous buffer. The SPAAC reaction on DNA has potential for applications in biology, genomics and nanotechnology.

AHE-S and TB have received funding from the European Union's Seventh Framework Programme (FP7/2007-2013) under grant agreement no 201418 (READNA).

Notes and references

1 P. Wu and V. V. Fokin, *Aldrichimica Acta*, 2007, **40**, 7–17.
2 A. H. El-Sagheer and T. Brown, *Chem. Soc. Rev.*, 2010, **39**, 1388–1405.
3 P. Kocalka, A. H. El-Sagheer and T. Brown, *ChemBioChem*, 2008, **9**, 1280–1285.
4 R. Kumar, A. El-Sagheer, J. Tumpane, P. Lincoln, L. M. Wilhelmsson and T. Brown, *J. Am. Chem. Soc.*, 2007, **129**, 6859–6864.
5 A. H. El-Sagheer, R. Kumar, S. Findlow, J. M. Werner, A. N. Lane and T. Brown, *ChemBioChem*, 2008, **9**, 50–52.
6 J. Gierlich, G. A. Burley, P. M. E. Gramlich, D. M. Hammond and T. Carell, *Org. Lett.*, 2006, **8**, 3639–3642.
7 F. Seela and X. Ming, *Helv. Chim. Acta*, 2008, **91**, 1181–1200.
8 F. Seela, V. R. Sirivolu and P. Chittepu, *Bioconjugate Chem.*, 2008, **19**, 211–224.
9 S. Berndl, N. Herzig, P. Kele, D. Lachmann, X. H. Li, O. S. Wolfbeis and H. A. Wagenknecht, *Bioconjugate Chem.*, 2009, **20**, 558–564.
10 F. Seela, H. Xiong, P. Leonard and S. Budow, *Org. Biomol. Chem.*, 2009, **7**, 1374–1387.
11 F. Seela and V. R. Sirivolu, *Chem. Biodiversity*, 2006, **3**, 509–514.
12 A. H. El-Sagheer and T. Brown, *J. Am. Chem. Soc.*, 2009, **131**, 3958–3964.
13 H. Isobe, T. Fujino, N. Yamazaki, M. Guillot-Nieckowski and E. Nakamura, *Org. Lett.*, 2008, **10**, 3729–3732.
14 T. Fujino, N. Yamazaki and H. Isobe, *Tetrahedron Lett.*, 2009, **50**, 4101–4103.
15 A. H. El-Sagheer and T. Brown, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 15329–15334.
16 J. C. Jewett, E. M. Sletten and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2010, **132**, 3688–3690.
17 A. H. El-Sagheer and T. Brown, *Pure Appl. Chem.*, 2010, **82**, 1599–1607.
18 Z. J. Gartner and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 4606–4610.
19 P. V. Chang, J. A. Prescher, E. M. Sletten, J. M. Baskin, I. A. Miller, N. J. Agard, A. Lo and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 1821–1826.
20 S. T. Laughlin, J. M. Baskin, S. L. Amacher and C. R. Bertozzi, *Science*, 2008, **320**, 664–667.
21 S. T. Laughlin and C. R. Bertozzi, *ACS Chem. Biol.*, 2009, **4**, 1068–1072.
22 N. J. Agard and C. R. Bertozzi, *Acc. Chem. Res.*, 2009, **42**, 788–797.
23 J. C. Jewett and C. R. Bertozzi, *Chem. Soc. Rev.*, 2010, **39**, 1272–1279.
24 M. F. Debets, C. W. J. van der Doelen, F. Rutjes and F. L. van Delft, *ChemBioChem*, 2010, **11**, 1168–1184.
25 X. Ning, J. Guo, M. A. Wolfert and G. J. Boons, *Angew. Chem., Int. Ed.*, 2008, **47**, 2253–2255.
26 G. Groger, U. Behrens and F. Olbrich, *Organometallics*, 2000, **19**, 3354–3360.
27 R. T. Ranasinghe and T. Brown, *Chem. Commun.*, 2005, 5487–5502.
28 P. van Delft, N. J. Meeuwenoord, S. Hoogendoorn, J. Dinkelaar, H. S. Overkleeft, G. A. van der Marel and D. V. Filippov, *Org. Lett.*, 2010, **12**, 5486–5489.
29 K. N. Jayaprakash, C. G. Peng, D. Butler, J. P. Varghese, M. A. Maier, K. G. Rajeev and M. Manoharan, *Org. Lett.*, 2010, **12**, 5410–5413.
30 A. H. El-Sagheer, V. V. Cheong and T. Brown, *Org. Biomol. Chem.*, 2011, **9**, 232–235.