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Efficient reverse click labeling of azide oligonucleotides with multiple alkynyl Cy-Dyes applied to the synthesis of HyBeacon probes for genetic analysis

Marta Gerowska, Lucy Hall, James Richardson, Montserrat Shelbourne, Tom Brown *

School of Chemistry, University of Southampton, Highfield, Southampton SO17 1BJ, UK

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ABSTRACT

A convenient method of oligonucleotide labeling using click chemistry has been developed. A 2′-mesyloxyethyl ribothymidine phosphoramidite monomer was incorporated into DNA at several loci during solid phase oligonucleotide synthesis and converted to 2′-azidoethyl ribothymidine in high yield on the synthesis resin. The resultant azide oligonucleotides were doubly and triply labeled with alkyne-modified cyanine dyes and their biophysical properties were studied. The influence of the dye structures and method of labeling on the fluorescence properties of the DNA probes is discussed and compared with a standard labeling method using active esters of Cy-Dyes.

1. Introduction

Fluorescence remains the principal detection method in DNA diagnostics, sequencing, genomics, and forensic analysis.[1,2] Fluorescent probes have found wide applications in the detection of genetic variations in DNA and identification of single nucleotide polymorphisms. The most commonly used probes are Molecular Beacons,[3–7] Scorpion primers,[8,9] TaqMan probes,[10,11] Hairpin primers,[12] and Hybridization probes.[13] Currently there is a drive to develop novel fluorescence techniques to provide highly sensitive and rapid nucleic acid interrogation methods that are compatible with high throughput sequence analysis. Continual improvements in nucleic acid labeling chemistry and fluorophore design are vital to support these developments, and the new technologies require a supply of dyes with varying fluorescence characteristics.[14] Among the most widely employed fluorophores in nucleic acid-based applications are the cyanine dyes. They are used as donors/acceptors in fluorescence resonance energy transfer (FRET) to measure distances in biological systems,[15–17] in nucleic acids sequencing, in fluorescence in situ hybridization (FISH), in DNA microarrays,[18–20] and for bio-imaging in living cells.[21–25]

Two main strategies are used for the attachment of fluorophores to oligonucleotides; addition during solid-phase synthesis, and post-synthetic labeling. The first method requires the availability of phosphoramidite monomers with the dye attached to an artificial backbone, a sugar or a nucleobase. This approach is not feasible if the dye is unstable to oligonucleotide deprotection conditions. The second strategy, post-synthetic labeling, is accomplished by reaction of an active ester derivative of a fluorophore with an amino-modified oligonucleotide. Although this method is more widely employed than any other oligonucleotide labeling strategy, it is low yielding when multiple fluorophore additions are required. An alternative labeling chemistry, the copper-catalyzed 1,3-dipolar cycloaddition reaction between a terminal alkyne and an azide (CuAAC reaction), has become an important method for attachment of reporter groups to nucleic acids, peptides, sugars, and other biomolecules.[26] The term ‘click chemistry’ was introduced to describe this and other highly efficient and selective chemical reactions.[27–30] The CuAAC reaction for the post-synthetic labeling of alkyne-modified DNA has been reported by Carell,[31,32] Seela[33] and others,[26] and has recently been used for fluorescent labeling of uridine using phenoxazinium-azide and coumarin-azide.[34] There are many examples of labeling alkyne-modified oligonucleotides with azide-functionalized dyes, but there are only limited reports of the use of alkyne-derivatized fluorophores and azide labeled oligonucleotides. Seo et al. successfully labeled a 5′-azide oligonucleotide with an alkyne derivative of fluorescein in the absence of Cu(I), but this method required large excess of the dye, high temperature and long reaction times.[35] Lietard et al. described

* Corresponding author. Tel.: +44 (0) 2380 592974; fax: +44 (0) 2380 592991; e-mail address: tb2@soton.ac.uk (T. Brown).

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a method for the introduction of an azide into the 5′-end of the oligonucleotide by coupling bromohexenyl phosphoramidite to the oligonucleotide and converting the bromo group to azide.[36] Both methods, despite being efficient, are restricted to labeling at the 5′-end. Recently, Aigner et al. described the synthesis of the 2′-azido-2′-deoxyuridine and 2′-azido-2′-deoxyadenosine-3′-phosphodiester monomers for incorporation into RNA by manual phosphotriester coupling to the RNA strand.[37] This approach avoided the undesired reaction between an azide and P(III) in the same molecule.[38] The same group has synthesized 3′-azido-modified tRNA using a 3′-azido-3′-deoxyadenosine-functionalized solid support for the synthesis of RNA,[39] and Wagenknecht has reported the conversion of 5-iodo-dU to 5-azido-dU in oligonucleotides on a solid support.[40]

We have been exploring simple and efficient methods of introducing multiple azide groups into oligonucleotides in order to carry out 'reverse click labeling' with reporter groups, and in this paper we describe a suitable method for achieving this objective. We also describe the convenient synthesis of a series of alkyne-functionalized cyanine dyes (Fig. 1) and their use in labeling HyBeacon probes. The fluorescent labels are attached to the 2′-position of the ribose sugar of thymidine and do not prevent base pairing. Our approach can be used to introduce fluorophores onto any thymidine nucleotide in a DNA sequence, and for multiple labeling. Reverse click labeling is particularly useful for functionalizing oligonucleotides with Cy-Dyes, given the availability of a wide range of ethynyl Cy-Dyes. The reverse-click oligonucleotide labeling methodology is outlined below and the synthesis of the ethynyl Cy-Dyes is described in the Supplementary data.

2. Results and discussion

2.1. Synthesis of azide-functionalized oligonucleotides and dye-labeling

Our dye-labeling method is described in Fig. 2. In the first step the 2′-mesyloxyethyl ribothymidine[41,42] is converted to a phosphoramidite monomer, which is introduced into oligonucleotides during solid-phase synthesis. The mesyl group is then displaced by azide while the DNA remains attached to the solid support. This 'mesyl to azide' conversion strategy was adopted because of the well-known incompatibility of the azide functionality with P(III) chemistry (Staudinger reaction).[43,44] Although it is possible to successfully carry an azide group through oligonucleotide synthesis (e.g., using 3′-azide attached to a resin)[39] it is not possible to combine the azide group with P(III) *in the same monomer*, hence azides cannot be used as phosphoramidite monomers and this limits the synthesis of oligonucleotides containing multiple azides. In contrast to azide, the mesyl group is fully compatible with phosphoramidite oligonucleotide synthesis. Suitable conditions for the azide displacement reaction were found to be sodium azide in anhydrous DMF with a reaction time of 20 h at 65 °C, under which the azide displacement step proceeds in high yield (Fig. 3A).

The dye-labeling reactions were carried out on the oligonucleotide synthesis resin to take advantage of the ease by which the excess free dye can be removed by washing the solid support with organic solvents. A tris-hydroxypropyl triazole ligand was used to accelerate the reaction and prevent oligonucleotide degradation.[45] The copper(II) sulfate and sodium ascorbate provided a source of Cu(I). The ethynyl Cy-Dyes were dissolved in DMSO and the

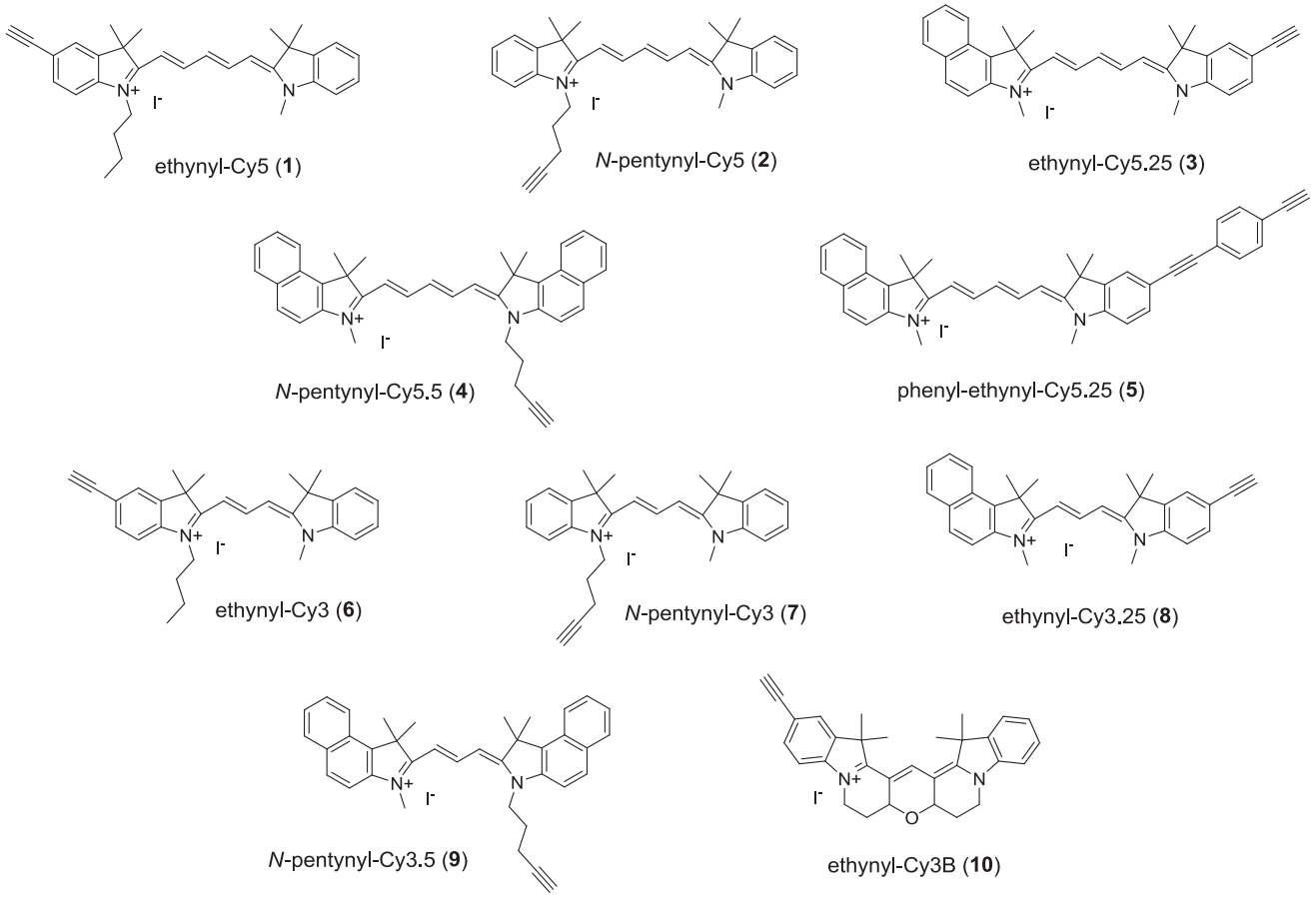

Fig. 1. Structures of Cy5, Cy3, and Cy3B derivatives synthesized in this study; ethynyl-dyes (Et), *N*-pentynyl-dyes (Np), and phenyl-ethynyl-dye (Ph).