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A new approach to oligonucleotide labelling using Diels–Alder cycloadditions and detection by SERRS†

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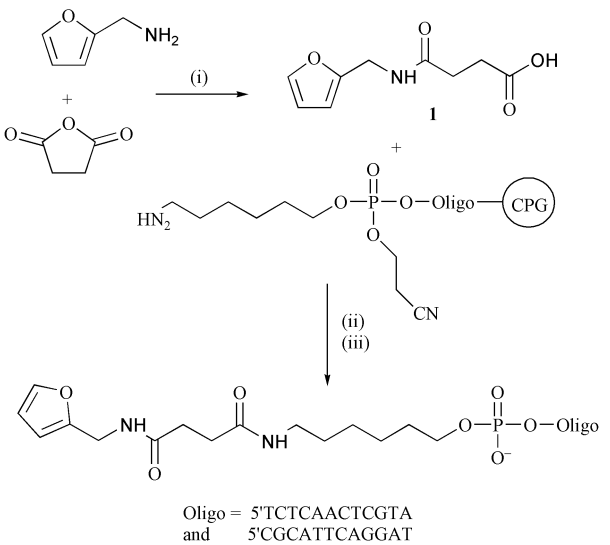
Received (in Cambridge, UK) 17th May 2002, Accepted 29th July 2002
First published as an Advance Article on the web 8th August 2002

Diels–Alder cycloaddition has been used to add a benzotriazole azo dye to a diene tagged oligonucleotide to generate unique SERRS signals at 10 attomoles.

Modern DNA detection makes use of a number of physical techniques that utilize detection of a label covalently added to an oligonucleotide probe. In particular fluorescence spectroscopy is widely used.[1,2] The addition of an external label such as a fluorophore can be a disadvantage since most labels have appreciable biological activity in that they can associate with particular biomolecules in a non-covalent fashion. This can lead to reduced and in some cases total probe inactivity.[3,4] Additionally any excess labeled probe has to either be removed from the assay or internally masked prior to analysis. This leads to extra separation procedures or the use of complicated and expensive probes. In an attempt to overcome these problems we have designed a new approach to detection based on Diels–Alder cycloadditions and surface enhanced resonance Raman scattering, SERRS which provides unique signals of the desired product using a simple probe without use of separation steps at ultra low concentrations (Scheme 1).

SERRS requires the presence of a coloured moiety that will adsorb onto a metal surface.[5,6] This produces enhanced Raman scattering at ultra low concentration levels and with a high degree of molecular specificity.[7,8] In this new approach a small chemical tag is added to a probe molecule. The tag is invisible to the detection technique of SERRS and is only made SERRS active by use of a developing agent that reacts specifically with the tag to produce the label. As the new species has a different molecular structure, the signal produced is unique to that species and shows differences to that of the developing reagent. Unlike fluorescence, this enables detection without separation.

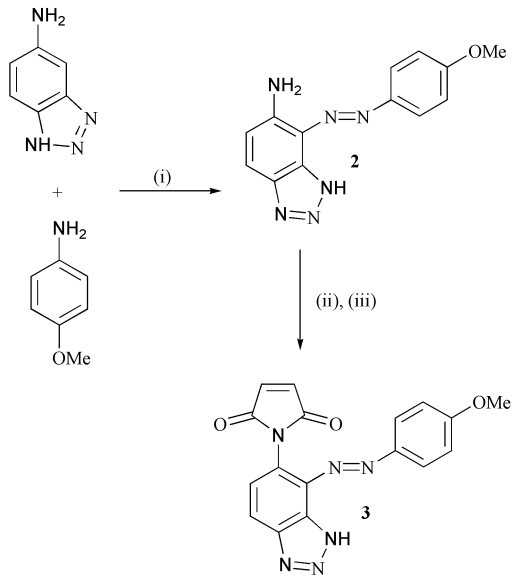


Scheme 1 *Reagents and conditions*: (i) DCM, (ii) CDI, DMF, 40 °C, (iii) NH_4OH, 55 °C.

Diels–Alder cycloaddition was chosen as the chemistry for labeling, as it is a fast, quantitative reaction that occurs in aqueous solution. A diene was used as the tag and a dienophile as the developing reagent. Diels–Alder cycloaddition has been used previously to add fluorescent labels to oligonucleotides highlighting the simplicity and ease of the approach for direct labeling but unlike our approach isolation of the labeled probe was required prior to analysis.[9] In this study the approach of using Diels–Alder cycloaddition to produce a SERRS active species is demonstrated by using oligonucleotides. The diene chosen was furan which has been shown to react with a maleimide to produce different SERS signals[10] and is easily added to the 5'-terminus of a synthetic oligonucleotide (Scheme 1). (This method has been used to prove the principle of cycloaddition. Synthesis of a furan phosphoramidite is currently under investigation to allow more efficient addition.) Two different oligonucleotides were used to ensure that there were no sequence specific effects from subsequent experiments.

A new, non-fluorescent dye was designed as the developing agent using a maleimide as the dienophile, an azo and a benzotriazole group to complex to the silver surface. (Scheme 2).

The synthesis of a benzotriazole dye maleimide (BDM), which incorporated the maleimide as part of the chromophore so that the SERRS spectrum of the cycloadduct was different from the BDM, was a substantial synthetic challenge. A number of alternatives were investigated before the route shown in Scheme 2 was chosen. The most difficult step was the intramolecular cyclisation to form the maleimide. A number of standard methods were attempted, however, due to low yields or



Scheme 2 *Reagents and conditions*: (i) HCl, $NaNO_2$, NaOAc pH 6.0, (ii) MalAn, DCM, (iii) Co naphthenate, DMA, Ac_2O.

† Electronic supplementary information (ESI) available: full experimental details on the synthesis and analysis of the reported compounds. See http://www.rsc.org/suppdata/cc/b2/b204790j/

DOI: 10.1039/b204790j

side reactions the method of choice was the use of cobalt(II) naphthenate with acetic anhydride.[11]

The oligonucleotides were then exposed to the BDM (**3**) under a variety of conditions and the reaction initially followed at high concentration by HPLC to ensure the reaction was occurring. The optimal conditions were found to be similar to those reported by Hill *et al*.[9] except that the reaction time and number of equivalents of dienophile required could be reduced by use of a copper(II) nitrate catalyst. Equimolar amounts of furan oligo and BDM could be used to give complete reaction with the catalyst. It also reduced the reaction time to less than one hour. Both oligonucleotides gave the same result which proved that the reaction was not a sequence dependent process. Fig. 1 shows the cycloaddition as followed by HPLC using a 25 mM phosphate buffer at pH 5.5 and a three-fold excess of BDM.

The reaction was then followed by SERRS at a concentration lower than could be detected by a UV-HPLC system to evaluate the sensitivity. In this case a 1:1 ratio of BDM to furan oligonucleotide was used and no separation steps were performed prior to analysis. Two methods of SERRS analysis were investigated. One was the addition of 10 μl of the reaction mixture to a silver film produced *in situ* from a silver nitrate/PVA matrix and the other was the addition to citrate reduced silver colloid. The PVA films have been used previously to give good SERRS[12] and in this case direct deposition of the mixture followed by an aqueous wash after 10 min resulted in the excellent spectra shown in Fig. 2. The cycloadduct showed distinct differences from the BDM. The spectra were more intense, new bands formed at 1204, 1263 and 1387 (azo stretch) cm^{-1} and there were changes in relative intensity in several other bands. The most obvious features to be used for detection are the shift in frequency of the band at 1399 to 1387 cm^{-1} and the change in intensity of the band at 1434 cm^{-1} on production of the cycloadduct. DFT on related compounds indicated that these bands are predominantly stretching modes of the aromatic rings with a contribution from the azo stretch.

When the silver colloidal suspension was used, direct addition of the mixture to the suspension resulted in very poor signals. However, as found previously for oligonucleotides, addition of spermine allowed improved surface adsorption[13,14] and resulted in similar spectra to those obtained from the PVA films. Surprisingly the signal to noise ratio of the oligonucleotide cycloadducts is significantly better than that of the cycloadduct alone. We attribute this to the presence of the oligonucleotide influencing the surface adsorption properties of the cycloadduct. The sensitivity of these oligonucleotides is excellent, with the sensitivity of the PVA films being better than that of the colloid. The exciting light is focused onto a small area of the film (×50 objective d = 5 μm, ≈20 pm^2) and we estimate the actual number of moles under examination as being in the region of 10 attomoles.

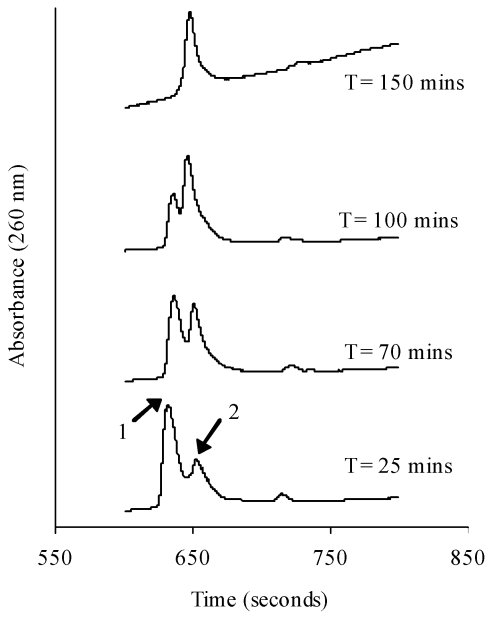


Fig. 1 HPLC trace of the cycloaddition. Peak 1 = furan oligo peak 2 = oligo cycloadduct.

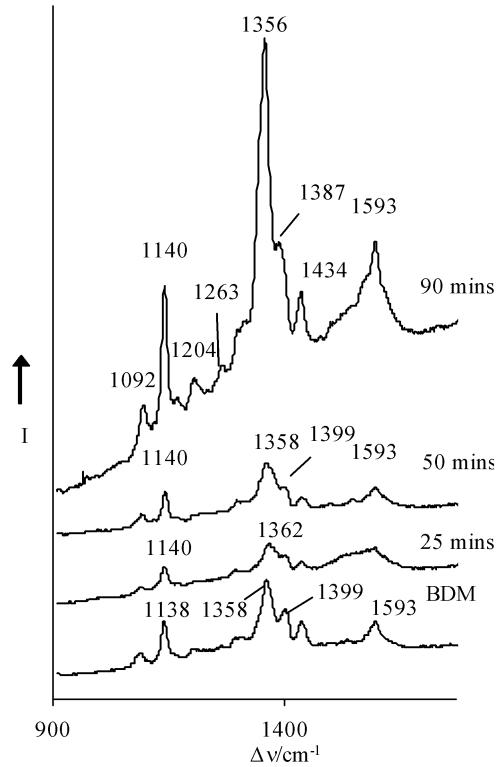


Fig. 2 SERRS of the cycloaddition from a silver film; 10 μl of a 6.3×10^{-8} M solution of reaction mixture was used for each analysis.

This is a totally new approach to biological labeling and shows excellent promise in terms of selectivity, sensitivity and simplicity. It is a convenient method of labeling oligonucleotides in an aqueous environment to produce distinctive species that do not need separation from starting materials prior to analysis. This approach to labeling of biomolecules will be effective for many other targets and a number of alternatives are under investigation.

The authors wish to the BBSRC for the award of a David Phillips Fellowship to D. G.

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