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SERRS detection of PNA and DNA labelled with a specifically designed benzotriazole azo dye†

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PNA and DNA have been detected for the first time with a specifically designed non-fluorescent SERRS active label; this is also the first use of SERRS to detect PNA.

In the post human genome project era a significant effort will centre on the detection of known nucleic acid sequences and variations within those sequences. Currently most methods of achieving this detection rely on the use of specific molecular biological assays and a physical detection technique which selectively identifies a label in a sensitive and discriminatory manner. Fluorescence and radio labelling are the most widely used physical techniques.[1,2] Recently we have reported the detection of labelled oligonucleotides using surface enhanced resonance Raman scattering, SERRS.[3–5] The main benefits of using SERRS rather than fluorescence are the ability to discriminate between labels in a mixture, without separation, at femtomole levels or below and the more extensive, simpler labelling chemistry which can be employed.[4]

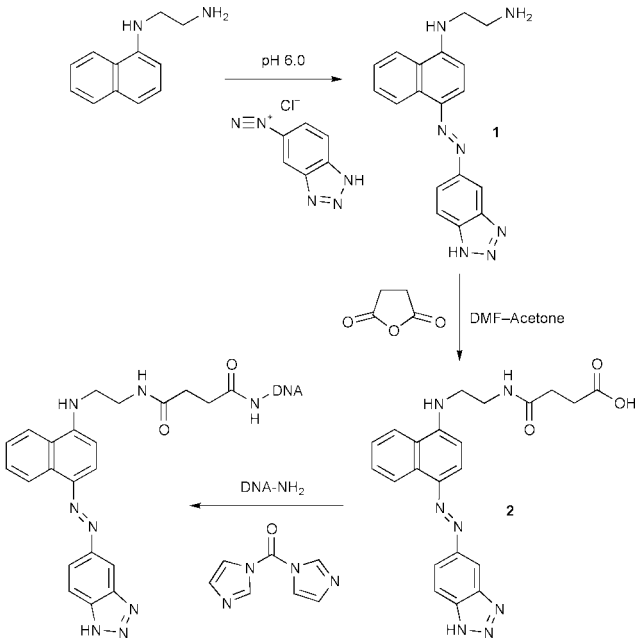
To obtain SERRS a dye requires to be adsorbed onto a roughened surface of certain metals of which the most widely used are silver and gold. The dye is required to obtain molecular resonance enhancement and the surface attachment provides enhancement by interaction with the plasmons on the metal surface. The combined enhancement processes provide a Raman signal of equivalent sensitivity to that of fluorescence. The sharp vibrational signals provide much better selectivity and since many dyes which are not fluorophores are effective, the chemistry can be much simpler and more extensive. A very effective metal surface used in this laboratory is that of citrate reduced silver colloid.[6] Aggregating agents such as sodium chloride or acid provide the most effective roughened surface and tune the surface plasmon to match the frequency of the excitation used.[7] In all previous SERRS studies involving DNA we have used commercially available fluorophores as the labels, as fluorescence is quenched in SERRS thus allowing the enhanced light scattering to be observed. Surface adsorption has been by electrostatic attraction of positively charged ammonium groups on the label to negatively charged citrate groups on the surface of the metal colloid used in our studies. In this approach the adsorption is not robust and can be affected by the presence of other agents such as metal chelators and no use is made of the extensive additional chemistry available for use with SERRS methodologies.

Previously we reported the synthesis of benzotriazole monoazo dyes specifically designed to provide SERRS by complexing directly to the silver surface, through the benzotriazole moiety which displaced the citrate surface layer.[8] In this communication we report the synthesis of a derivative of one of the previous dyes which is capable of undergoing solid phase addition to both DNA and PNA to make the oligomers SERRS active. The benefits of using these labels are that they are easy to produce, they give distinctive SERRS signals which are different from the previously used fluorophores, and they bind *via* complexation to the silver surface in a way which produces virtually irreversible binding.

The route followed for synthesis of the dye is shown in Scheme 1. *N*-(1-Naphthyl)ethylenediamine was chosen due to the presence of the primary aliphatic amine. Once an azo linkage has been produced the electron density on the aromatic amine is pulled into the ring system[9] dramatically reducing the reactivity of the amine as a nucleophile. Thus the aliphatic amine provides a reactive functionality to further derivatise the dye once synthesised. Dye synthesis was achieved by a diazonium coupling using 5-aminobenzotriazole to produce the orange dye, *N*-[4-(5′-azobenzotriazolyl)naphthalen-1-yl]ethylenediamine [**1**]. This dye was further functionalised to produce *N*-[2-(4′-(5″-azobenzotriazolyl)naphthalen-1-yl)aminoethyl]-succinamic acid [**2**]. The acidic dye was then capable of addition to amino linked DNA and PNA *via* amide formation on the solid phase.

A DNA oligomer was synthesised by routine solid phase phosphoramidite chemistry[10] and a monomethoxytrityl protected amino link added to the 5′-terminus.[11] Removal of the monomethoxytrityl group and addition of the activated dye produced the 5′-dye labelled DNA on the solid phase. The dye was activated by addition of carbonyl diimidazole to form the active ester. Cleavage and deprotection produced the crude labelled oligonucleotide which was purified by ion exchange HPLC. The coupling efficiency was estimated as > 83% by integration of the peaks from the HPLC.

PNA has a N-terminus which can be used to react in the same way as the amino link in DNA, however two Fmoc protected amino-3,6-dioxaoctanoic acid (AEEA-OH) spacers[12] were



Scheme 1 Synthesis of the benzotriazole label and addition to amino linked DNA.

† Electronic supplementary information (ESI) available: full experimental details. See http://www.rsc.org/suppdata/cc/b1/b102241p/

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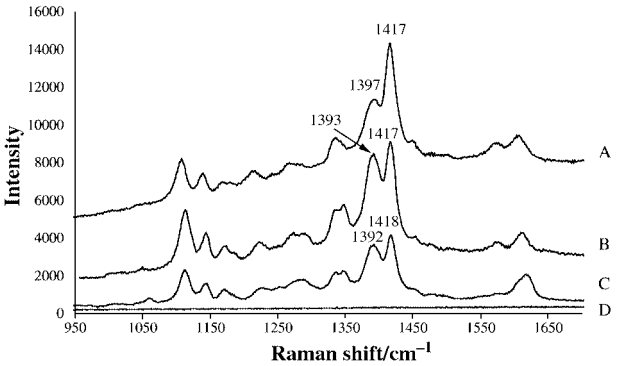

Fig. 1 The SERRS spectra obtained for free dye (A), dye labelled DNA (B), dye labelled PNA (C) and aggregated colloid with no dye (D). The spectra were obtained under different conditions and are scaled to allow comparison.

added to distance the dye from the PNA sequence. An 8mer was synthesised using Fmoc/Bhoc chemistry[12] and the two spacers added to the N-terminus. Removal of the Fmoc protecting group from the terminal spacer allowed addition of the benzotriazole carboxylic acid dye in the same way as for the DNA. Use of the dye as a monomer in the PNA synthesis cycle resulted in a mixture of products and poor coupling due to the unprotected triazole system, hence the use of the carbonyl diimidazole. Deprotection yielded the crude oligomer as a purple solid due to protonation of the azo linkage. Purification by reverse phase HPLC gave the pure dye labelled PNA with an estimated coupling efficiency of ~55% for the dye. The low coupling could be from a number of sources, such as cross-reaction of the unprotected triazole group. However, as only one monomer was being added this yield was acceptable.

The oligomers were then investigated for their ability to produce SERRS using citrate reduced silver colloid. A Renishaw microprobe system was used with 1 mW of 514.5 nm excitation at the sample. The optimum conditions for obtaining SERRS of both benzotriazole labelled oligomers vary. For the DNA oligomer the highest signal intensity was obtained when the phosphate backbone was neutralised by the addition of spermine prior to addition to the silver colloid. Use of an excess of spermine also provides aggregation of the colloid to produce the desired SERRS signals.[4] The spectrum is shown in Fig. 1B. The spectrum is consistent with that of the uncoupled dye (Fig. 1A) and displays the stretches at ~1393 cm^{-1} and 1417 cm^{-1} that are indicative of an azo tautomer.

A citrate assay was used to monitor the release of citrate from the surface after the addition of the benzotriazole dye, to the colloid. This proved that citrate was released from the surface after addition of the benzotriazole dye, but if spermine was added alone to induce aggregation then citrate was not released. This indicates that the benzotriazole is indeed complexing directly with the metal and not by a charge–charge interaction which appears to occur during spermine aggregation. Additionally, SERRS signals can be obtained from the dye labelled oligonucleotide without the use of spermine by using alternative aggregating agents such as phosphate buffer or nitric acid. This is in direct contrast to the oligonucleotides labelled with the fluorophores which in previous studies only produced intense SERRS with spermine. Thus we can conclude that the benzotriazole dye acts as a surface complexing agent and displaces citrate to provide attachment of modified oligonucleotides to a silver surface.

The conditions for obtaining SERRS of the PNA oligomer differ in that the oligomer is dissolved in 0.l% TFA and has a neutral backbone. Thus there is no need for the use of spermine as a charge neutralising agent. Also the TFA acts as an aggregating agent, again negating the need for the addition of spermine as an aggregating agent. The addition of the labelled PNA oligomer to a colloidal suspension produced a strong and distinct SERRS spectrum identical to that obtained for the labelled DNA (Figure 1C). In order to investigate the SERRS of the PNA labelled oligomer, a set of standard aggregating agents was tested with the labelled 8mer and the resulting SERRS recorded (Table 1). The values show that the intensity of the signals for the labelled PNA can be improved by the addition of an aggregating agent. However, if the solution is made basic by the addition of triethylamine then the intensity of the signals decreases, indicating that the PNA provides optimal signal to noise ratios in acidic conditions which are compatible with the conditions used for the standard synthesis of PNA. Spermine still appears to enhance the intensity of the spectrum, as it is an efficient aggregating agent.

Table 1 The intensity of the major peak in the PNA spectrum with different aggregating agents. All spectra were recorded in one scan of ten seconds and at 2×10^{-11} mole equivalents

Species studied	Intensity of peak at 1418 cm^{-1}
Colloid + TFA	No peaks
Colloid + BtDYE PNA + triethylamine	866
Colloid + BtDYE PNA	1074
Colloid + BtDYE PNA + spermine	1269
Colloid + BtDYE PNA + NaCl	2341
Colloid + BtDYE PNA + TFA	4148
Colloid + triethylamine	No peaks

In conclusion, we have synthesised benzotriazole azo dye labelled oligomers specifically designed to give SERRS. This dye is not an effective fluorophore and gives an indication of the additional labelling chemistry available for use with SERRS. The dye has been added to both DNA and PNA and SERRS obtained from both species. This type of label has additional benefits since the covalently attached benzotriazole group gives strong bonding, particularly to silver and copper surfaces, and provides a new and effective DNA–metal bonding chemistry. This is the first time that SERRS has been obtained from PNA and it is clear that it is easier to obtain SERRS from PNA than DNA.‡§

Notes and references

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§ Oligomers synthesised were: DNA 5′-BtDye X GTG CTG CAG GTG TAA ACT TGT ACC AG 3′ (X = amino link) and PNA (N)-BtDye OO ACA TTT GA (C) (O = AEEA spacer).

1 L. J. McBride and M. D. Oneill, *Am. Laboratory*, 1991, **23**, 52 *et seq*.
2 A. Castro and J. G. K. Williams, *Anal. Chem.*, 1997, **69**, 3915.
3 D. Graham, W. E. Smith, A. M. T. Linacre, C. H. Munro, N. D. Watson and P. C. White, *Anal. Chem.*, 1997, **69**, 4703.
4 D. Graham, B. J. Mallinder and W. E. Smith, *Biopolymers (Biospectroscopy)*, 2000, **57**, 85.
5 D. Graham, B. J. Mallinder and W. E. Smith, *Angew. Chem., Int. Ed.*, 2000, **39**, 1061.
6 C. H. Munro, W. E. Smith, M. Garner, J. Clarkson and P. C. White, *Langmuir*, 1995, **11**, 3712.
7 C. Rodger, W. E. Smith, G. Dent and M. Edmondson, *J. Chem. Soc., Dalton Trans.*, 1996, 791.
8 D. Graham, C. McLaughlin, G. McAnally, J. C. Jones, P. C. White and W. E. Smith, *Chem. Comm.*, 1998, 1187.
9 D. Graham, A. R. Kennedy and S. J. Teat, *J. Heterocycl. Chem.*, 2000, **37**, 1555.
10 M. H. Caruthers, *Science*, 1985, **230**, 281.
11 S. L. Beaucage and R. P. Iyer, *Tetrahedron*, 1993, **49**, 1925.
12 R. Castle, I. S. Jensen and M. Egholm, in *Peptide Nucleic Acids: Protocols and Applications*, ed. P. E. Nielsen and M. Egholm, 1999.