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THE PREPARATION OF ENZYME-LABELLED OLIGONUCLEOTIDES BY REDUCTIVE AMINATION

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Abstract. Oligonucleotides containing an aldehyde group at the 3'-terminus have been prepared using a derivatized solid phase. This solid phase contained a short peptide that included a lysine residue substituted at the N^{ε}-amino group with carboxybenzaldehyde. The oligonucleotide was coupled to alkaline phosphatase under reductive amination conditions to produce a conjugate with unaltered hybridization properties.

Enzyme conjugates of oligonucleotides have been used widely as nonradioactively labelled probes. An advantage is that the catalytic label is attached directly to the oligonucleotide. Thus the label can be detected without the need for extra steps that may introduce artefacts, as is the case in the indirect detection of biotin-containing oligonucleotides using avidin or streptavidin conjugates.

Various methods have been reported for the preparation of enzyme-containing oligonucleotides. In general, a modified oligonucleotide containing either an amino or a thiol group is prepared, followed by coupling to the enzyme using a bifunctional coupling agent. The first such conjugates were reported by Jablonski *et al.*, who used an oligonucleotide containing a single modified nucleotide with a 12-atom linker arm terminating in a primary aliphatic amine[1]. This oligonucleotide was reacted with the heterobifunctional reagent disuccinimidyl suberate (DSS), the intermediate activated oligonucleotide then purified and reacted with alkaline phosphatase (AP), utilising the second succinimidyl ester from DSS now present on the oligonucleotide. The final product was purified (30 - 50% overall yield) and was found to have retained full enzymatic activity. The conjugate had a Tm (melting temperature of the duplex with a complementary unmodified strand) that was lower than that of the unmodified oligonucleotide by approximately 10 °C. The hybridization kinetics of these probes were also tested, and they were found to hybridize slower than their ^{32}P-labelled counterparts[2]. The second order rate constants were 3.6×10^5 $mol^{-1}s^{-1}$ for an AP-labelled 53mer and 5.8×10^5 $mol^{-1}s^{-1}$ for the same probe labelled with ^{32}P. Probes prepared by this approach have been also used in the technique of hybridization histochemistry to detect specific mRNA species in tissue sections[3a-d]. Farmar and Castaneda developed a similar, albeit simpler protocol for preparing oligonucleotide-enzyme conjugates[4]. Li *et al.* used a different approach, utilising a 5'-aminoalkyl-modified oligonucleotide which was reacted with an intermediate reagent to introduce a thiol group, and then reacted with a bromoacetyl-derivatized AP[5a]. The reported conjugation yield was 80%. The conjugate retained 60% of the activity of the enzyme. These probes were successfully used to detect enterotoxigenic *E. Coli* isolates that had been blotted on a membrane[5b]. Urdea *et al.*[6] used a similar method to Jablonski *et al.* They reacted the aminoalkyl-modified oligonucleotide with *p*-phenylene diisothiocyanate (DITC), extracted with *n*-butanol to remove the excess DITC and then reacted the resulting oligonucleotide with the enzyme without further purification. They used both horseradish peroxidase

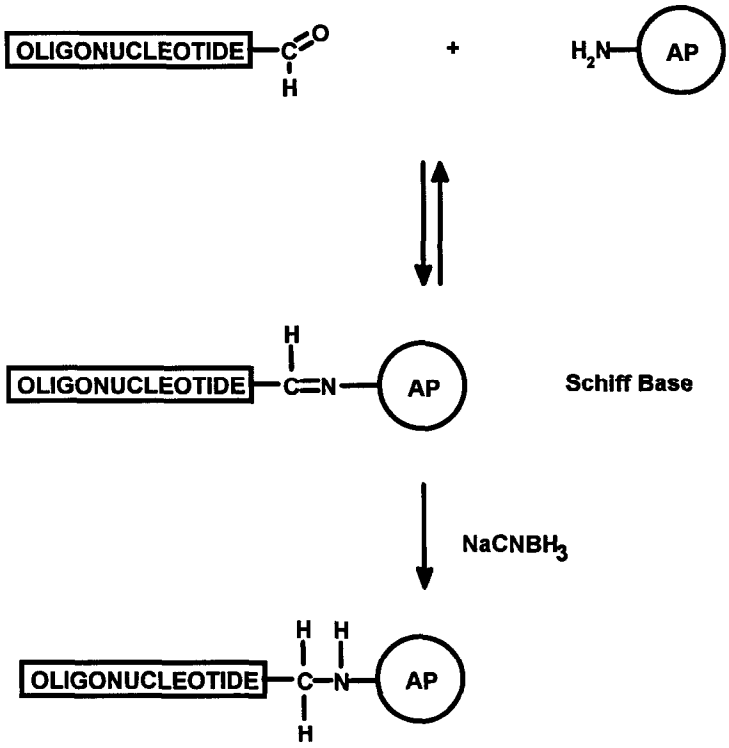
(HRP) and AP. The conjugates were purified by polyacrylamide gel electrophoresis (PAGE). With an optimal assay for detection of the enzyme, the sensitivity of the probes approached that of 5'-^{32}P labelled probes Other heterobifunctional reagents have been also used to prepare enzyme conjugates, such as *m*-maleimidobenzoyl-N-hydroxysuccinimidyl ester (MBS)[7a], the avidin-biotin reaction[7a], and N-hydroxysuccinimidyl 3-(2-pyridyldithio)propionate (SPDP)[7b]. Commercial systems are also available for producing AP-labelled probes. The E-LINK™ kit from Cambridge Research Biochemicals (CRB, Northwich, UK) uses an aminoalkyl-modified oligonucleotide which is reacted with an unspecified cross-linking reagent to produce an activated oligonucleotide. This is followed by reaction with an activated form of AP supplied with the kit. Probes prepared by this method have been used to detect propiomelanocortin (POMC) mRNA in tissue sections[8]. The LIGHTSMITH™ system from the Promega Corporation (Madison, WI) use similar aminoalkyl-modified oligonucleotides, which are also reacted with a heterobifunctional reagent and, after reduction with dithiothreitol (DTT), are reacted with an activated AP[9a-b]. The sensitivity limit of this system, when AP is detected with the optimal chemiluminescent detection system[9b,10] is reported to be approximately 3 attomole.

Previously we have prepared conjugates of oligonucleotides with enzymes such as AP and HRP with the use of crosslinking agents such as DSS and DITC. In our hands, the conjugations with DSS were low yielding and conjugations with DITC, while giving a respectable yield of the conjugate, resulted in conjugates with low enzymic activity. In this paper, we report on a new method for conjugating oligonucleotides to proteins, making use of reductive amination coupling. The advantage of this method is that is does not require an intermediate reaction with a heterobifunctional reagent prior to the actual coupling of the oligonucleotide with the enzyme, since the oligonucleotide is in a form that can be reacted directly with the enzyme.

Reductive amination has been used in the past for the coupling of large molecules possessing appropriate reactive groups[11]. In this methodology, a molecule having an aldehyde group is reacted with another molecule containing a primary aliphatic amino group. The intermediate product is the imine (Schiff base), which is in equilibrium with the starting materials. A reducing agent is then used to convert the imine into the secondary amine, thus making the reaction irreversible and driving it to completion. We decided to utilise this reaction to couple an oligonucleotide to an enzyme. The oligonucleotide contains an aldehyde group, and this reacts with primary aliphatic amino groups present in the enzyme (ε-amino groups of lysine residues and the *N*-terminus, Scheme I). Aldehyde-containing oligonucleotides had been previously prepared, by post-synthetic methods[12]. The aldehyde in this work is introduced into the oligonucleotide as part of a modified lysine residue in an oligonucleotide-peptide conjugate.

Controlled Pore Glass (CPG) resin was derivatized as previously reported to contain a protected primary aliphatic hydroxy group linked to the resin through a spacer[13]. Peptide synthesis was carried out using the Fmoc (fluorenylmethoxycarbonyl) methodology[14]. An alanine residue was introduced as the first amino acid, followed by a lysine and then two aminohexanoic acid (εAhx) spacers. The resin was then treated with 90% TFA / 10% ethanedithiol (5 min) to remove the Boc protecting group on the lysine (Scheme II). Carboxybenzaldehyde was coupled to the free side chain amino group using ten equivalents of each of the aldehyde, 1-hydroxybenzotriazole (HOBt) and BOP reagent (benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluoroborate), and fifteen equivalents of N-methylmorpholine (NMM). The N-terminal Fmoc group was then removed (20% piperidine in DMF, 5 min), and the linker molecule **1** added, to convert the

SCHEME I

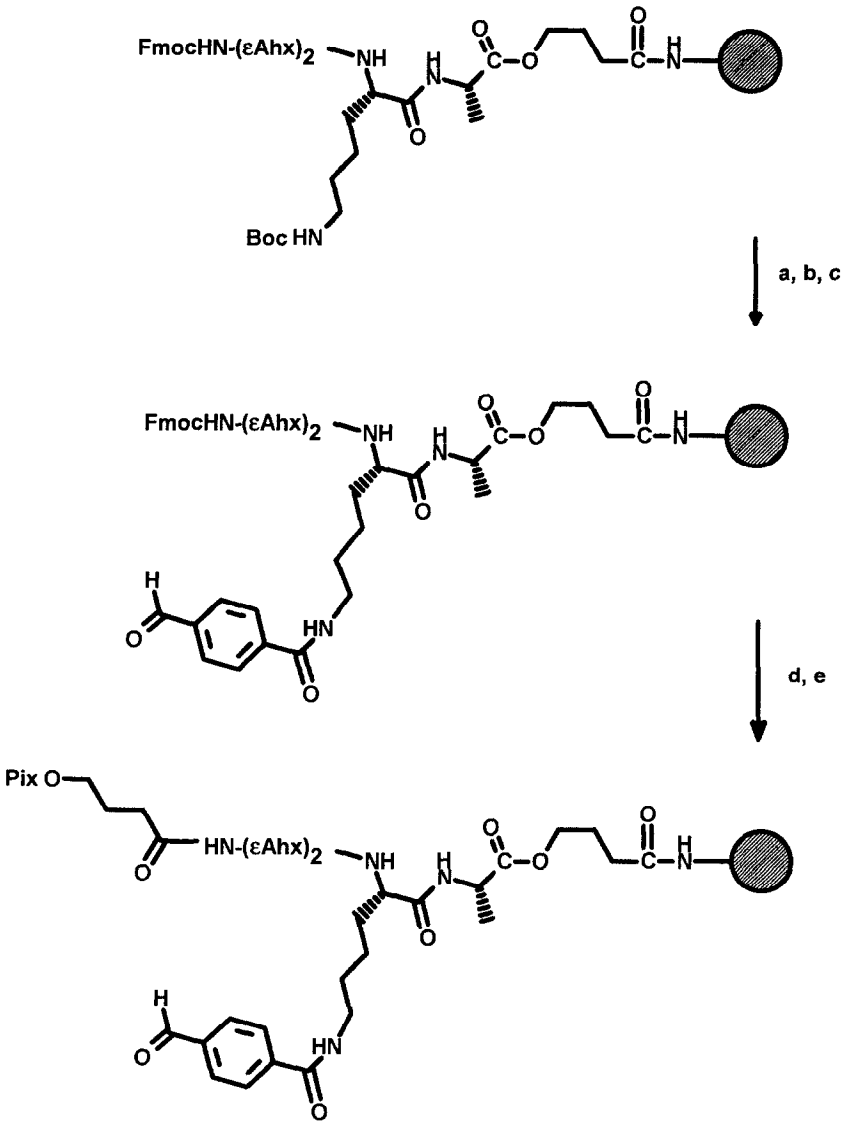


terminus to an pixyl (9-phenylxanthen-9-yl)-protected hydroxyl group. This substrate was then used for oligonucleotide synthesis on the ABI 380A Automated DNA Synthesizer using standard methods[13].

The oligonucleotide synthesized on this solid support was GGGCTTCACAACATCTGTGATGTC-AGCAGG (KPIB), complementary to a region of mouse kallikrein mRNA[13]. The cleavage from the solid support and deprotection was carried out using 0.1 M NaOH rather than concentrated ammonia[15]. The crude oligonucleotide was purified by reverse phase HPLC. The broad, late-eluting peak was collected.

The aldehyde-containing oligonucleotide was conjugated to AP by a direct coupling, using sodium cyanoborohydride as the reducing agent. The oligonucleotide (7.2 nmol) was lyophilized in an Eppendorf tube. This was dissolved with the AP solution (Boehringer Mannheim calf intestine AP for enzyme immunoassay, cat no. 567 744, 0.5 mg, 3.6 nmol, in the buffer supplied by the manufacturer). Sodium cyanoborohydride solution in water (0.02 mg, 0.32 μmol, in 1 μl, 90 equiv each time) was added every hour for five hours. The reaction mixture was then allowed to stand overnight. The extent of reaction was determined by SDS-PAGE. Alkaline phosphatase is a dimeric enzyme with a monomeric molecular weight of approximately 70 KDa. Addition of the

SCHEME II



a) 90% TFA / 10% ethanedithiol, 5 min; b) 20% Et_3N / CH_2Cl_2; c) carboxybenzaldehyde, HOBt, BOP, NMM; d) 20% piperidine / DMF; e) *O*-pixyl 4-hydroxybutyric acid *p*-nitrophenyl ester (**1**), HOBt

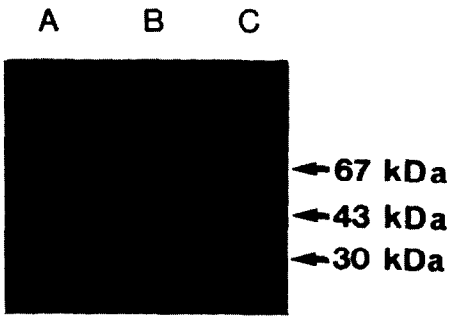


Figure 1. SDS-PAGE of the conjugation reaction mixture, after silver staining. Lane A, conjugate; lane B, unreacted AP; lane C, molecular weight markers.

oligonucleotide adds approximately 10 KDa to a subunit. Two bands are seen for the reaction product on SDS-PAGE, one for the conjugated subunit (slow running) and one for the subunit without an attached oligonucleotide (Figure 1). The ratio of these two bands gives an indication of the extent of labelling, which appears to be high (a ratio of one corresponds to one oligonucleotide molecule per enzyme). A possible third, higher molecular weight band is probably due to a subunit having two oligonucleotide molecules attached. Excess oligonucleotide was removed by repeated ultrafiltration through a membrane with a molecular weight cutoff of 50,000. Spectrophotometric determination of the amounts of enzyme and oligonucleotide present, using the absorbances at 260 and 280 nm with the determined extinction coefficients of the enzyme and oligonucleotide at these wavelengths gave a ratio of oligonucleotide to enzyme of 1.35 : 1. The overall yield of purified product was 53%, based on the amount of AP recovered. The activity of the conjugate was determined using a fluorescent method, with 4-methylumbelliferyl phosphate as substrate[16]. This showed that the enzyme retained 65% of its activity after the conjugation, considerably higher than we were able to achieve using previously published conjugation methods.

The ability of the conjugate to hybridize to its complementary nucleic acid was tested by spectrophotometric Tm measurements. The conjugate and its complementary oligonucleotide were dissolved in a buffer suitable for hybridization experiments with alkaline phosphatase labelled oligonucleotides, and a DNA melting experiment was carried out using a Varian Cary 1 temperature-controlled spectrophotometer, using a ramp rate of 0.25 °C per minute. The Tm of duplexes formed between the complementary oligonucleotide and one of the following oligonucleotides were determined[17]: (i) the unmodified KPIB oligonucleotide, (ii) the enzyme-labelled oligonucleotide. These were found to be 50.3 ± 0.4 °C and 48.9 ± 0.6 °C for (i) and (ii) respectively, indicating that the presence of the enzyme decreased the Tm by only 1.4 °C. This compares with the 10 °C difference reported for conjugates prepared by the DSS method. In addition, we have shown recently

that these conjugates will hybridize to kallikrein mRNA in tissue sections of mouse salivary glands (data to be published elsewhere).

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References and Notes

1. Jablonski, E.; Moomaw, E. W.; Tullis, R. H.; Ruth, J. L. *Nucleic Acids Res.* **1986**, *14*, 6115
2. Podell, S.; Maske, W.; Ibanez, E.; Jablonski, E. *Mol. Cell. Probes* **1991**, *5*, 117.
3. (a) Baldino, Jr., F.; Ruth, J. L.; Davis, L G. *Exp. Neurol.* **1989**, *104*, 200; (b) Kiyama, H.; Emson, P. C.; Ruth, J *Eur. J. Neurosci.* **1990**, *2*, 512; (c) Kiyama, H.; Emson, P. C ; Ruth, J. L, Morgan, C. *Mol. Brain Res.* **1990**, *7*, 213, (d) Kiyama, H.; Emson, P C. *J. Histochem. Cytochem.***1991**, *39*, 1377.
4. Farmar, J. G.; Castaneda, M. *BioTechniques*, **1991**, *11*, 588
5. (a) Li, P.; Medon, P. P.; Skingle, D. C.; Lanser, J. A.; Symons, R. H. *Nucleic Acids Res.* **1987**, *15*, 5275; (b) Medon, P. P.; Lanser, J. A.; Monckton, P. R.; Li, P.; Symons, R. H *J. Clin. Microbiol.* **1988**, *26*, 2173
6. Urdea, M. S.; Warner, B. D.; Running, J. A.; Stempien, M.; Clyne, J.; Horn, T. *Nucleic Acids Res.* **1988**, *16*, 4937.
7. (a) Alves, A. M.; Holland, D.; Edge, M. D.; Carr, F. J. *Nucleic Acids Res.* **1988**, *16*, 8722; (b) Murakami, A.; Tada, J.; Yamagata, K.; Takana, J. *Nucleic Acids Res.* **1989**, *17*, 5587.
8. McNicol, A. M.; Farquharson, M. A.; Walker, E. *Path. Res. Pract.* **1991**, *187*, 556.
9. (a) McCormick, M.; Mendoza, L., Wu, L.; Abdel-Mawgood, A.; Lewis, K.; Smith, C. E *Promega Notes* **1992**, *37*, 1; (b) McCormick, M.; York, C.; Wu, L.; Simpson, D.; Smith, C. *Promega Notes* **1993**, *40*, 4.
10. Schaap, A. P.; Akhavan, H.; Romano, L. *Clin. Chem.* **1989**, *35*, 1863.
11. Gray, G. R. *Methods Enzymol.* **1978**, *50*, 155.
12. Kremsky, J. N.; Wooters, J. L., Dougherty, J. P.; Meyers, R. E.; Collins, M.; Brown, E. L. *Nucleic Acids Res.* **1987**, *15*, 2891.
13. Haralambidis, J.; Duncan, L.; Angus, K.; Tregear, G. W. *Nucleic Acids Res.* **1990**, *18*, 493.
14. Atherton, E.; Sheppard, R. C. *Solid Phase Peptide Synthesis: A Practical Approach*; IRL Press at Oxford University Press: Oxford, **1989**.
15. Dreyer, G. B.; Dervan, P. B. *Proc. Natl. Acad. Sci. USA* **1985**, *82*, 968.
16. Rietz, B.; Guibault, G. G. *Clin. Chem.* **1975**, *21*, 1791.
17. The hybridization solution used in all of the Tm measurements had the following composition: 40% formamide, 0.02% Ficoll, 0.02% bovine serum albumin, 0 02% polyvinylpyrollidone, 0.6 M NaCl. This solution was found to have little effect on the specific activity of the enzyme.

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