The preparation of polyamide-oligonucleotide probes containing multiple non-radioactive labels

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ABSTRACT

Oligonucleotide probes containing multiple non-radioactive labels have been prepared by utilising and extending the methods used to prepare polyamide-oligonucleotide conjugates. The probes were prepared by incorporating suitable amino acid residues, such as lysines, in the polyamide, which were then used as sites for the attachment of the non-radioactive labels. The procedures developed give control over the distance of the label from the oligonucleotide, and also the inter-label distance. The labels can be conveniently introduced while the substrate is still on the solid support. Even though fluorescent oligonucleotide probes prepared in this way carrying multiple carboxyfluorescein labels gave low levels of fluorescence due to quenching, the probes containing ten biotin labels gave a detection sensitivity of approximately 5 attomole (3 million molecules).

INTRODUCTION

The preceding paper in this issue¹ describes the preparation of oligonucleotides containing a 3'-polyamide moiety. These modified oligonucleotides have the potential to be used as probes carrying multiple non-radioactive labels. The derivatization of oligonucleotides to produce very sensitive non-radioactive probes is an area which has received considerable attention recently¹. The compounds that we have developed lend themselves to this application since the polyamide can be designed as a multi-functional entity that can be used to carry multiple non-radioactive labels. In evaluating this approach we chose two types of commonly used non-radioactive labels: a fluorophore (fluorescein) and biotin.

The mouse kallikrein genes were used as a test system. The kallikreins are a series of processing enzymes, and this system has been extensively investigated at both the cellular and genetic level in our laboratory². The biotin-labelled probes prepared by the method described here gave a higher signal intensity than probes previously reported containing a single biotin label and are of similar sensitivity to enzyme labelled probes³.

RESULTS

Fluorophore labelling of the conjugates

There are a number of stages in the procedure for the synthesis of polyamide oligonucleotide conjugates¹ at which non-radioactive labels can be added: the label can be present in the Lys synthon before polyamide synthesis, it can be introduced after polyamide synthesis (stage (i)), after addition of the linker (stage (ii)), after oligonucleotide synthesis (stage (iii)) on the solid support, or after deprotection and purification of the product (stage (iv)) (Figure 1). The method chosen will depend on the choice of label and synthetic procedure. We have attached fluorophores to the polyamide by removal of the *tert*-butoxycarbonyl (Boc) protecting groups on the lysine residues after polyamide or oligonucleotide synthesis and subsequent reaction with the fluorogenic synthon 1, a non-fluorescent protected carboxyfluorescein (CF) derivative. This compound can be prepared using standard methods and only becomes fluorescent upon deprotection of the phenolic hydroxyls which takes place during the ammonia treatment. An additional advantage of using this compound is that, since it will not become fluorescent until the final deprotection step, there is less likelihood of photochemical bleaching of its fluorescence during the synthetic procedures.

A number of fluorophore-containing oligonucleotides were prepared by these methods, introducing the label at stages (i), (ii) or (iii) (Table I). These compounds were only partially purified by passage through a column of Sephadex G-25 and subsequent dialysis of the high molecular weight fraction. It was immediately obvious that the addition of multiple fluoresceins did not result in a greater amount of fluorescence as these compounds were highly coloured, but not very fluorescent. The relative fluorescence of the conjugates containing multiple fluorophore-bearing lysine residues with a variable number of ϵ Ahx spacer residues between the lysines is shown in Table I. The extent of labelling of the single fluorescein containing conjugate was 40%, those of the ϵ Ahx containing conjugates quantitative and that of the oligolysine compound 65%. However, at least in the case of the compound with double ϵ Ahx spacers, a decrease in the extent of labelling to 50% did not result in a significant change to the relative fluorescence per fluorescein moiety. The extent of labelling was calculated by determining

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Figure 1. Flow diagram of the synthetic protocol. Non-radioactive labels can be added at the stages marked (i)–(iv), or can be incorporated prior to solid-phase synthesis in a labelled amino acid.

Table I. Fluorescence yields for various fluorescein containing polyamide-oligonucleotide conjugates. Excitation and emission were at 495 and 525 nm respectively, with 2.5 nm monochromator slits.

	Relative Fluorescence/CF 495/525
Carboxyfluorescein (CF)	1
KPIB-SL-(ϵ Ahx) ₄ Lys(CF)-Ala	0.92
KPIB-SL-Ala-Lys(CF)-[(ϵ Ahx) ₄ Lys(CF)] ₉ Ala	0.05
KPIB-SL-[(ϵ Ahx) ₂ Lys(CF)] ₁₀ Ala	0.06
KPIB-SL-[(ϵ Ahx-Lys(CF))] ₁₀ Ala	0.015
KPIB-SL-Ala-[Lys(CF)] ₂₀ Ala	0.002

the absorbance of the solution at pH 9 at 260 nm and 495 nm. The absorbance of the fluorescein moieties at 260 nm was taken into account when determining the concentration of the oligonucleotide. The conjugate containing a single fluorescein gave a comparable amount of fluorescence per fluorescein moiety to carboxyfluorescein itself. This result contrasts with that reported previously, in which an oligonucleotide labelled with fluorescein attached *via* an arm to the N-4 of cytosine was only 20% as fluorescent as fluorescein⁴. However, as the density of the fluorescein moieties increases, the relative amount of fluorescence drops dramatically. The amount of fluorescence per fluorescein moiety is 20 times less than that of CF in the conjugates with ten lysines and two or four ϵ Ahx spacers between the lysines, and this decreases to 70 times less for the single ϵ Ahx spacer and 500 times less for the oligolysine compound.

Figure 2. Dot blot hybridization results using biotinylated conjugates. Each dot contains 1 μ g of herring sperm DNA and the indicated amount of the pUC plasmid containing a kallikrein cDNA insert. The negative control contains 1 μ g of a pUC plasmid containing an unrelated insert. The conjugates used are: lane A, KPIB-SL-[(ϵ Ahx)₄Lys(Biotin)-Ala], labelled at stage (iv); lane B, KPIB-SL-[(ϵ Ahx)₂Lys(Biotin)-Ala], stage (ii); lane C, KPIB-SL-[(ϵ Ahx)₂Lys(Biotin)]₁₀Ala, stage (iii); lane D, KPIB-SL-[(ϵ Ahx-Lys(Biotin))]₁₀Ala, stage (iii); lane E, KPIB-SL-[(ϵ Ahx)₂Lys(Biotin)]₁₀Ala, stage (i). Hybridization and detection is described in the text.

Biotin labelling of the conjugates

A number of biotinylated oligonucleotides were prepared, containing either one or ten biotin residues. Addition of the biotin at stage (i) was readily accomplished using underivatized biotin and utilizing the BOP (benzotriazol-1-yl-oxy-tris(dimethylamino)phosphonium hexafluoroborate) coupling methodology⁵. Alternatively, biotin can be introduced as the N-hydroxy-succinimidyl active ester, at any of the stages described. Figure 2 shows the results obtained when singly or multiply labelled oligonucleotides were used to hybridize to dot blots containing a pUC plasmid with a mouse kallikrein cDNA insert. The signal was detected by using the BRL Blugene kit, which employs a streptavidin-alkaline phosphatase conjugate that binds to biotin, and a subsequent development reaction in which the enzyme acts on substrates to generate an insoluble dye precipitate.

In lanes A and B (Figure 2), hybridization was carried out using singly labelled biotinylated conjugates. The conjugate used in lane A was labelled at stage (iv) and that in B at stage (ii). It is evident that the conditions of oligonucleotide synthesis do not adversely affect the ability of the biotin residue to bind to the streptavidin conjugate. The observation that the signal in lane B (limit of sensitivity 50 pg of plasmid) is in fact a little stronger than that in lane A (100 pg) could be explained by the fact that the conjugate in lane A was not purified further after the solution

phase biotinylation reaction, and so may contain less biotin per conjugate molecule. The conjugates containing ten biotin residues (lanes C–E) give a signal that is considerably stronger than that in lanes A and B. The conjugates in lanes C and D were labelled at stage (iii), the conjugate in C having double ϵ Ahx spacers between the labelled lysines and that in D single ϵ Ahx spacers, and that in lane E at stage (i) (double ϵ Ahx spacers). These give similar signals, with lane E being a little stronger (10 pg) than the other two (20 pg). The best conjugate with ten biotin labels is thus five times more sensitive than the best conjugate with a single label, and there does not appear to be a significant difference between the multi-labelled conjugates with single or double ϵ Ahx spacers. The sensitivity of the probe in lane E is equivalent to 4.4 amol (2.6 million molecules) of plasmid.

DISCUSSION

Addition of non-radioactive labels can be accomplished at a number of stages during the synthesis of the polyamide-oligonucleotide conjugates. The most useful approach, is when the label is added while the conjugate is only partially deprotected and still on the solid support. A large excess of label can then be used to drive the reaction to completion with unreacted reagent being simply washed from the solid support. If desired the label can be incorporated in the amino acid prior to polyamide synthesis. The advantage of this methodology is that a large number of labels can be added to an oligonucleotide probe with total synthetic control of the disposition of the labels. The number of labels to be added is not limited by the number of nucleotide residues in the oligonucleotide.

The fluorophore-containing conjugates prepared by this method were highly fluorescent when a single fluorescein moiety was attached but only a small amount of fluorescence was present in multiply labelled conjugates. The amount of fluorescence in fluorescein-labelled conjugates containing ten lysine residues also decreased further as the distance between the fluorophores was decreased. The most likely mechanism for this quenching of the fluorescence is non-radiative energy transfer—a through space dipole-dipole interaction^{6,7}. This can occur since there is considerable overlap between the excitation and emission spectra of fluorescein, leading to an interaction between an excited molecule and a nearby molecule in the ground state. It would not be unexpected for this to occur given the high local concentration of fluorescein moieties present in the conjugates. The Forster distance R_0 , at which the probability of transfer is one half, has been measured for fluorescein and found to be in the order of 5 nm⁸. This is approximately the same as the distance between the fluoresceins in the compound with the four ϵ Ahx spacers between the lysine residues, assuming that the spacers are rigid. In practice however, because of the highly flexible spacers, the average distance between fluorophores will be much less. Furthermore, the absorption spectra of the conjugates show that there is a considerable amount of ground state dimer formation (data not shown), and the dimers are known to be better energy traps than the ground state monomers⁸. With a large number of nearby acceptors to which the excitation can be transferred increasing the probability of transfer, the low amount of fluorescence found is not surprising.

It was relatively easy to add any number of biotin residues to suitable conjugate molecules. The conjugates containing ten biotin residues gave a signal that was approximately five times stronger than those containing a single biotin residue, with the size of the

spacers (one or two ϵ Ahx residues) between the label carrying lysine residues having no significant effect. It thus appears that the high local concentration of biotin residues reduces their ability to be detected by the enzyme conjugate only by a factor of two.

In conclusion, we have developed a synthetic procedure that can be used to synthesize conjugate polyamide-oligonucleotide molecules of a variety of molecular architectures and containing variable numbers of different non-radioactive labels. Even though our multi-fluorescein labelled probes were only weakly fluorescent, further experiments are underway to develop oligonucleotide probes containing multiple fluorophore residues that are highly fluorescent. However, we were able to prepare multi-biotin labelled probes which gave a much greater signal than single biotin labelled probes. This demonstrates the utility of preparing multi-labelled probes using this methodology.

EXPERIMENTAL

¹H and ¹³C NMR spectra were recorded on a Bruker AM300 at 300 and 75 MHz respectively with tetramethylsilane as internal reference.

Dimethylformamide (DMF) was distilled under reduced pressure and used within 1–2 days. Pyridine was distilled from potassium hydroxide and stored over 5A molecular sieves. Biotin N-hydroxysuccinimide ester was prepared by the method of Becker *et al.*⁹ Microanalyses were determined by Amdel, Melbourne. Melting points were determined in open-ended capillaries on an Electrothermal Melting Point Apparatus. Spectrofluorometric measurements were carried out on a Perkin-Elmer LS-5 Luminescence Spectrometer. Flash chromatography¹⁰ was carried out using Merck Kieselgel # 9385.

Spiro[isobenzofuran-1(3H),9'-[9H]xanthene]-5(and -6)-carboxylic acid, 3', 6'-(2-methylpropanoyloxy)-3-oxo, pentafluorophenyl ester (1)

Trimellitic anhydride (9.6 g, 0.05 mol) and resorcinol (11 g, 0.11 mol) were mixed thoroughly and placed in a 190°C oil bath for 1 h. The temperature was then increased to 210°C, and was kept there for 5 h, by which time the melt had solidified to a dark red solid. It was then allowed to cool down and dissolved in 50 mL of DMF. To this solution was added 100 mL of pyridine and isobutyl chloride (15.4 mL, 0.15 mol). This was allowed to stir for 24 h, was filtered, the filtrate concentrated to a thick syrup, was redissolved in 400 mL of EtOAc, and washed with 1 M H₂SO₄ (2×300 mL) and H₂O (1×300 mL), dried (Na₂SO₄), and again concentrated to a syrup. It was redissolved in CH₂Cl₂ and flash chromatographed on a silica gel column (170 g), eluting first with CH₂Cl₂ (800 mL) and then with 2% MeOH/CH₂Cl₂. The fractions containing the product were pooled and the solvent removed *in vacuo* to give 12.45 g (48%) of crude diisobutyl-5(and -6)-carboxyfluorescein.

5.77 g of this material (11 mmol) and pentafluorophenol (2.23 g, 12.1 mmol) were dissolved in 125 mL of 1/1 EtOAc/CH₂Cl₂. The solution was cooled down to 4°C, and DCC (2.5 g, 12.1 mmol) was added dropwise. It was allowed to stir for 2 h, was then filtered and the solvent removed *in vacuo* to give an oil, which solidified overnight at –20°C. This was redissolved in 50 mL of CH₂Cl₂ and flash chromatographed on 150 g of silica gel, eluting with CH₂Cl₂. The fractions containing the product were pooled, the solvent removed *in vacuo* and the product recrystallized from 95% EtOH/2% Acetic acid, to give 3.68 g

(59%) of white needles, mp 184–188°C. On tlc (CH_2Cl_2) this gave two closely running spots, corresponding to the two isomers, at R_f 0.58 and 0.63. ^1H NMR (CDCl_3) δ 1.31 (d, $J = 6.9$ Hz, 12H, CH_3), 2.8 (septet, $J = 7.0$ Hz, 2H, $\text{CH}(\text{CH}_3)_2$), 6.83 (s, 4H, H_1' and H_2'), 7.11 (s, 2H, H_4'), 7.38 (d, $J = 8.0$ Hz, 0.5H, H_7 of 5-isomer), 7.96 (s, 0.5H, H_7 of 6-isomer), 8.20 (d, $J = 8.0$ Hz, 0.5H, H_6 of 5-isomer), 8.5 (m, 1H, H_4 and H_5 of 6-isomer), 8.87 (s, 0.5H, H_4 of 5-isomer). ^{13}C NMR (CDCl_3) δ 18.82, 34.18, 82.11, 82.24, 110.61, 115.07, 115.11, 118.00, 125.01, 125.89, 126.36, 127.05, 128.08, 128.71, 129.28, 130.92, 132.23, 133.30, 136.32, 137.05, 138.2, 139.62, 141.8, 142.84, 151.46, 151.51, 152.66, 153.41, 158.21, 161.06, 167.50, 174.94. Anal. Calcd for $\text{C}_{35}\text{H}_{23}\text{F}_5\text{O}_9$: C, 61.6; H, 3.4. Found: C, 61.5; H, 3.0.

Since this material was analytically pure and was the material used in the fluorescein labelling experiments, it was used to determine the spectral characteristics of carboxyfluorescein (CF). A 1 μM solution of 1 in 0.1 M NH_4OAc , pH 9.0 was allowed to stand for 3 d to decompose 1 to the CF nucleus, and spectral measurements taken. The extinction coefficient of this solution at 495 and 496 nm was 75500 M^{-1} , and at 260 nm 23500 M^{-1} . Since the extinction coefficient of the released pentafluorophenoxide ion in the same solution at 260 nm is 1300 M^{-1} , then the extinction coefficient of CF at 260 nm is 22200 M^{-1} . This solution was also used as the standard reference for the fluorescence yields of fluorescein containing polyamide-oligonucleotide conjugates.

Preparation of labelled conjugates

Conjugates were prepared as described in the preceding paper¹. Labels were introduced at four different stages: (i) after polyamide synthesis; (ii) after addition of the linking synthon; (iii) after oligonucleotide synthesis; and (iv) after deprotection and purification of the conjugate.

Stage (i) Biotin and fluorescein synthons were attached following removal of the Boc protecting groups on the lysine side chains by a five minute treatment with 90% trifluoroacetic acid/10% ethanedithiol. Biotin was coupled using a 0.2 M solution of each of biotin, BOP reagent, 1-hydroxybenzotriazole (HOBt) and N-methylmorpholine in DMF for 30 min. The degree of coupling was determined by quantitative ninhydrin test by following the disappearance of the reacting amino groups. For multiple lysine containing polyamides longer coupling times may be necessary. Biotin was also added by reaction with biotin N-hydroxy-succinimidyl active ester (3 equiv) and HOBt (3 equiv) in the minimum volume of DMF or dimethylsulphoxide (DMSO) for 16 hr. This coupling can also be carried out during polyamide synthesis after the addition of each Lys(Boc) residue. Fluorescein was coupled at this stage by reaction with a 0.3 M solution of 1 and HOBt in DMF for 3 d. Residual amino groups were acetylated. Since the subsequent piperidine treatment removes the isobutyryl protecting groups on the fluorescein, it was reacylated with DMAP/isobutyric anhydride in pyridine for 16 h, following the coupling of the linker.

Stage (ii) Coupling of label was carried out at this stage by utilizing ϵ -Fmoc (fluoren-9-ylmethoxycarbonyl) lysine side chain protection and Boc methodology for polyamide synthesis. Following removal of the ϵ -Fmoc group, biotin or fluorescein was added in the same manner as described in stage (i).

Stage (iii) The lysine side chain Boc protecting groups were removed as described previously¹ and the label attached. Biotin was added by reacting with biotin N-hydroxy-succinimidyl active ester (20 equiv) and HOBt (20 equiv) in DMF (0.5 mL), for 16 h. Fluorescein was added as in stage (i)—but reacylation of the fluorescein was not necessary.

Stage (iv) Biotin was added to the single lysine containing conjugate using standard methods (e.g. Agrawal *et al.*¹¹). Fluorescein can also be added with fluorescein isothiocyanate (FITC) by using standard methods (e.g. Smith *et al.*¹²).

Purification of Conjugates

The fluorescein containing conjugates were only partially purified, mainly to remove any free fluorescein and other low molecular weight material by passage through a column of Sephadex G-25 Fine (2 g) in H_2O . The high molecular weight fraction (0.5 mL) eluting at the void volume was collected and dialyzed (8000 molecular weight cutoff membrane) exhaustively against H_2O . Fluorescence measurements of the fluorescein containing conjugates were determined in 0.1 M NH_4OAc , pH 9.0, at concentrations where $A_{495} < 0.1$. The biotin containing conjugates were purified by polyacrylamide gel electrophoresis (PAGE)¹.

Nucleic acid Hybridization experiments

Hybridization experiments with the polyamide oligonucleotide (KPIB) conjugate probes were carried out by hybridizing onto dot blots containing a 3.7 kb plasmid derived from pUC and containing a 1 kb mouse renal kallikrein cDNA insert. The negative control was a similar pUC plasmid containing the metallothionein II_A gene promoter spliced with the chloramphenicol acetyl transferase (CAT) structural gene. Each dot contained 1 μg of herring sperm DNA as well. The nitrocellulose filters were prehybridized at 42°C for 6.5 h in 10 mL of hybridization buffer (0.75 M NaCl, 0.075 M sodium citrate, 25 mM NaH_2PO_4 , 25 mM Na_2HPO_4 , 10 mM tetrasodium pyrophosphate, 0.1 mM disodium adenosine triphosphate, 25 mg/L salmon sperm DNA, 0.01% w/v Ficoll, 0.01% polyvinylpyrrolidone, 0.01% bovine serum albumin, 20% formamide), the probe (100 ng) was then added and it was allowed to hybridize at 42°C overnight. The filters were then washed four times, for ten minutes each, at 42°C in 0.2 $\times$ SSC (0.03 M NaCl, 0.003 M sodium citrate). Detection of the biotin labelled probe was carried out with the BRL Blugene kit, following the manufacturer's instructions, and carrying out the colour development reaction for 3 h in the dark.

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