THE PHOTOTRITYL GROUP.
PHOTOCROSSLINKING OF OLIGONUCLEOTIDES TO BSA

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Abstract: A photoactivatable trityl cap was used to immobilize an oligonucleotide on BSA (bovine serum albumin). The conjugate was able to hybridize with a complementary sequence.

The immobilization of synthetic single stranded DNA to macromolecules is a recent topic in analytical biochemistry (diagnostic kits) and in the design of biologically active antisense oligonucleotides. Immobilized oligonucleotides may indeed be used to fish out a complementary sequence from a mixture of DNA or RNA. The coupling of oligonucleotide effectors to peptides, basic polymers and proteins enhances their cellular penetration or allows their targeting to specific tissues (e.g. asialoglycoprotein conjugates for targeting to hepatocytes).

The trityl protecting group has a remarkable retarding effect when oligonucleotides are purified by reversed phase HPLC. We reasoned that the trityl group could be used to anchor synthetic DNA fragments on hydrophobic surfaces. In order to render the adsorption irreversible, a hydrophobic crosslinking function had to be hooked to the trityl. We thus propose a novel method of immobilization of oligonucleotides to macromolecules based on a two step process: hydrophobic adsorption followed by photocrosslinking.

The synthesis of the required phototrityl group is sketched in the scheme. Briefly, diphenyl(m-cyanophenyl)carbinol 1 was reduced by LiAlH₄ in THF to diphenyl(m-aminomethylphenyl)carbinol 2 (67% yield). The amino function was acetylated to 3 by reaction with one molar equivalent of trifluoroacetic anhydride in pyridine. Refluxing with an excess of SOCl₂ in CH₂Cl₂ gave diphenyl(m-trifluoroacetylamino methylphenyl)chloro-methane 4 (100% yield). The readily accessible 1,2-dideoxy-D-ribofuranose 11 was selectively 5'-O alkylated by 4 (pyridine, 24h, R.T., 40% yield) to give 5 12, that was phosphitylated by a standard protocol and introduced as last reagent in a phosphoramidite-type automated DNA-synthesis. Concentrated ammonia (25%, 24h, 55°C) cleaved all the protections, including the trifluoroacetamide. Oligonucleotide 14 was acetylated by N-hydroxysuccinimidyl p-benzoylbenzoate, to give 8 (43% yield) 15. The trityl ether functions of 7 and 8 were fully stable in buffered solution at pH 7. They were however cleaved in water at pH 4, with half-lives of 16 h and 5 h, respectively (RT, 0.1 M citrate buffer, 0.02 M EDTA).

Albumin is well known for its capacity to bind hydrophobic drugs. Hydrophobic compounds, such as long-chain fatty acids (actually as their anions at physiological pH) are bound avidly to albumin. When a mixture of oligonucleotide 8 (10 μg) and an excess of monomeric BSA (2 molar equivalents, or more) in phosphate buffered saline (PBS = phosphate 12 mM, NaCl 138 mM, KCl 2.7 mM, pH 7.2) was ultrafiltered through a membrane of cutoff 30000 (Millipore, ULTRAFREE-MC30000PL filter units), the oligonucleotide was more than 90% retained by BSA. Without BSA, it passed through the membrane.
Scheme

1. OH

2. OH

3. X = COCF₃

4. X = COCF₃

5. NHCOCF₃

6. NHCOCF₃

7. X = H

8. X = CO

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a- LiAlH₄ / THF; b- CF₃COOCCF₃ / pyridine; c- SOCl₂ / CH₂Cl₂; d- 1,2-dideoxy-D-ribofuranose / pyridine; e- 2-cyanoethyl-N, N', N'-tetraisopropylphosphorodiamidite / diisopropylammonium tetrazolide / CH₂Cl₂; f- 1º) automated DNA-synthesis, 2º) conc. NH₃, 55º C, 24 h, 3º) N-hydroxysuccinimidyl p-benzoylebenzoate / borate buffer pH 9.
Oligonucleotides not bearing a trityl function were not captured by BSA. Moreover, both strands of a duplex of \( \text{cGATGACTG}_{3} \) and the complementary oligomer d(GTCGTGACTG\(_{3}\)A\(_{4}\)C) were retained, but \( \text{cGATGACTG}_{3} \) was the only strand captured when a mixture of \( \text{cGATGACTG}_{3} \) and the non-complementary oligomer d(CTCAGAGC\(_{5}\)GCCGCGAT) was equilibrated with BSA. The hydrophobic trityl cap thus allowed to anchor oligonucleotides on BSA. We then studied the photocrosslinking of \( \text{cGATGACTG}_{3} \) with this protein.

Oligonucleotide \( \text{cGATGACTG}_{3} \), dissolved in PBS (1\( \mu \)g/100 \( \mu \)l) in the presence of a large excess of BSA (25:1 molar ratio), was irradiated in a small glass tube by a slide projector for 7 hours (i.e. the time necessary for its complete disappearance). A conjugate BSA-oligonucleotide was isolated by preparative anion exchange FPLC\(^{18} \) and dialyzed against water (membrane cutoff 12000). Determination of its protein content (Coomassie blue binding method) allowed to calculate that 1 \( \mu \)mole of \( \text{cGATGACTG}_{3} \) gave 0.4 \( \mu \)mole of conjugate. It migrates just behind BSA in SDS PAGE\(^{19} \).

The conjugate (0.001 \( \mu \)mole), mixed with the complementary sequence (0.0005 \( \mu \)mole) in PBS (200 \( \mu \)l) at RT, captured the oligomer by hybridization (ULTRAFREE-MC30000PL ultrafiltration: 100% capture). The non-complementary oligomer d(CTCAGAGC\(_{5}\)GCCGCGAT) was not retained. This demonstrated that the sequence of \( \text{cGATGACTG}_{3} \) was accessible and not extensively degraded by photolysis.

We thus showed that the phototrityl group allows to immobilize an oligonucleotide in the hydrophobic pocket of a protein and that the immobilized oligomer is functional. The acid cleavage of trityl ethers by strong acids in organic solvents is well documented\(^{20} \). It is not generally recognized that they can be cleaved by dilute aqueous acids. Oligonucleotide-macromolecule conjugates captured by cells usually reach acidified intracellular compartments of pH about 5.5. We now realize that a phototrityl link could well be cleaved in such endosomes. The first generation phototrityl group described here is too slowly hydrolyzed in aqueous acid to be used for cell targeting, but electron-releasing substituents on the phenyl rings (e.g. p-methoxy) enormously increase the acid sensitivity of trityl ethers\(^{21} \). Such substituents could be easily introduced by using the same synthetic route as the one described here. This preliminary communication thus opens new perspectives in the cell targeting and intracellular release of synthetic DNA fragments.

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References and Notes

8. 300 MHz \(^{1}H\) NMR spectrum (CDCl\(_{3}\)): 7.0-7.4 (14H, aryls), 3.7 (s, 2H, CH\(_{2}\)), 2.3 (broad signal, 3H, OH and NH\(_{2}\)). 75 MHz \(^{13}C\) NMR spectrum (CDCl\(_{3}\)): 147.4, 147.0, 142.8, 127.9, 127.8, 127.1, 126.6, 126.4, 125.8 (aryl), 81.8 (C-OH), 46.3 (CH\(_{2}\)). Mass spectrum (E.I.), 289 (M\(^{+}\), 23%), 195 (loss of OH and C\(_{6}\)H\(_{5}\), 100%).
9. 300 MHz $^1$H NMR spectrum (CDCl$_3$): 7.2-7.3 (14H, aryls), 6.7 (1H, broad signal, NH), 4.4 (d, J = 5.8 Hz, 2H, CH$_2$), 3.0 (s, 1H, OH). 75 MHz $^{13}$C NMR spectrum (CDCl$_3$): 157.0 (q, C=O), 147.7, 146.5, 135.6, 128.5, 128.0, 127.9, 127.8, 127.4, 127.2, 126.6 (aryl), 116.0 (q, CF$_3$), 81.8 (C-OH), 43.8 (CH$_2$).

10. The only compound obtained by quenching of 4 by methanol was the corresponding methyl ether, $^1$H NMR spectrum: 7.5-7.1 (15H, aryls and NHCO), 4.5 (d, J = 5.7 Hz, 2H, CH$_2$), 3.0 (s, 3H, OCH$_3$).


12. 300 MHz $^1$H NMR spectrum (CDCl$_3$): 7.5-8.0 (15H, aryls and NHCO), 4.4 (2H, CH$_2$NH), 4.2, 4.1, 3.95 and 3.90 (4H, HOCH, CHOC and CH$_2$CH$_2$O), 3.1 (2H, trityl-O-CH$_2$), 2.1 and 1.8 (2x1H, CH$_2$CH$_2$O). 75 MHz $^{13}$C NMR spectrum (CDCl$_3$): 157.2 (q, C=O), 144.8, 143.4, 143.2, 135.8, 129.0-126.3 (aryl), 115.8 (q, CF$_3$), 86.4 (trityl C-O), 85.05 (CHOC), 73.5 (HOCH), 66.9 (CH$_2$CH$_2$O), 64.4 (trityl-O-CH$_2$), 43.6 (CH$_2$N), 34.7 (CH$_2$CH$_2$O).


14. Oligonucleotide 2 was isolated by C$_{18}$ reversed phase HPLC (see note 15). It was homogeneous in PAGE.

15. Typical conditions for the acylation of oligonucleotides by N-hydroxysuccinimidy 1-p-benzoylbenzoate: the acylating agent (2.8 mg, 8.7 μmole), dissolved in DMF (20μl) was added to oligonucleotide 2 (300 μg, 0.04 μmole), dissolved in 0.2 M borate buffer pH9 (180 μl). After 24 h, the turbid reaction mixture was centrifugated. The liquid phase was filtered (AcrodiscR 0.45 μm from Gelman Sciences) and analyzed by HPLC (BIOSIL C$_18$ HL 90 SS BIORAD, 40°C. Eluent A = triethylammonium acetate 0.1M, CH$_3$CN 5%. Eluent B = same, but CH$_3$CN 60%. Gradient from 5% B to 60% B in 1h. Flow rate 1 ml/min.). Oligonucleotide 2 featured a retention time of 26 min. and 8, a retention time of 42 min. Several peaks coming from the hydrolysis of the acylating agent were also present. They could be removed by ethanol precipitation of the oligonucleotide at -80°C (0.5 h). The acylated product 8 was isolated by prep-HPLC. It had the correct size in PAGE.


17. Bovine albumin was Cohn V fractionate (ethanol precipitated) from Sigma. The monomeric fraction was isolated by gel chromatography on a G-200 column of 2.5 cm width and 1 m length. The eluent was PBS. The oligonucleotide was incubated with BSA in PBS at 20°C for two hours. A complementary experiment, with compound 7, where X = 2-nitrobenzoyl instead of H, showed that trityl capped oligonucleotides were fully adsorbable on BSA. The percentage of oligonucleotide adsorbed (7, with X = 2-nitrobenzoyl, 1 μg of oligomer per 100 μl of PBS) was 61, 73, 89, 91, 92 and 96% at molar ratios oligonucleotide:BSA, 1:0.5, 1:1, 1:2, 1:5, 1:20 and 1:50, respectively.

18. Conditions of anion exchange FPLC: MONO Q HR 5/5 column from Pharmacia. Eluent A = TRIS 0.05M, pH8.6, CH$_3$CN 10%. Eluent B = same, but 2M NaCl. Gradient from 0% B to 100% B in 1h. Flow rate 0.5 ml/min. BSA had a retention time of 9 min and the conjugate had a retention time of 13 min.

19. The ExcelGel™ (gradient 8-18) precasted gel from Pharmacia was used as recommended by the supplier.


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