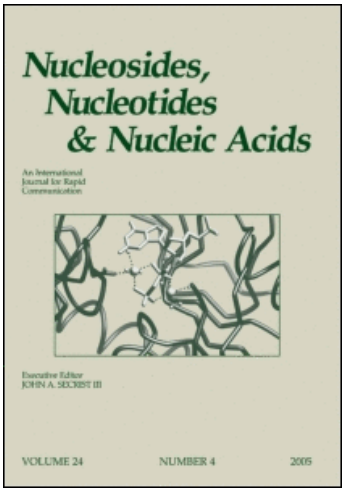

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Development of Suitable Linkage for Oligonucleotide Synthesis and Preliminary Hybridization Studies on Oligonucleotides Synthesized in Situ

A. Kumar[a]
[a] National Institute Of Immunology, New Delhi, INDIA

To cite this Article Kumar, A.(1994) 'Development of Suitable Linkage for Oligonucleotide Synthesis and Preliminary Hybridization Studies on Oligonucleotides Synthesized in Situ', Nucleosides, Nucleotides and Nucleic Acids, 13: 10, 2125 — 2134
To link to this Article: DOI: 10.1080/15257779408013212
URL: http://dx.doi.org/10.1080/15257779408013212

NUCLEOSIDES & NUCLEOTIDES, 13(10), 2125-2134 (1994)

Development of Suitable Linkage for Oligonucleotide Synthesis and Preliminary Hybridization Studies on Oligonucleotides Synthesized in Situ

AJAY KUMAR

National Institute Of Immunology,
Aruna Asaf Ali Marg,
New Delhi-110067, INDIA

ABSTRACT: The supports with disulphide and urethane linkage were prepared and their linkage stability was studied in ammonia solution. The urethane linkage was found to be more stable than the disulphide linkage. The simple hybridization with support linked oligonucleotide revealed the hybridization yields in the range of 10-15% . Interestingly, the triple helix hybridization yields were 92.4%,88.2%,72.5 %, 86.4% and 78.7% for polystyrene urethane, CPG (500^{O} A) urethane, CPG (3000^{O}A) urethane , (500^{O} A) disulphide and CPG (3000^{O}A) disulphide respectively.

INTRODUCTION

Dot hybridization (1) colony hybridization (3) and the hybridization technique developed by Southern (2) have been used in molecular biology. These methods are based on the non covalent immobilization of nucleic acids on matrices (4) such as nitrocellulose filter and nylon membranes. The low retention efficiency and relative inacessibility of the immobilized nucleic acid to the probing sequence , due to

only a small part of the target being available for hybridization , limits its potential sensitivity (5). The immobilized nucleic acid is not covalently attached , which can result in loss of nucleic acid from the support. These problems have been overcomed by using a mixed phase hybridization assay (6,7). In the sandwich type assay the target nucleic acid (analyte) hybridized to a oligonucleotide covalently immobilized on a solid support. A significant barrier to the more widespread use of DNA probe in the sandwich assays has been lack of suitable support of immobilization and suitable method for immobilization of oligonucleotide to the solid support.

The immobilization of DNA to the solid support has been carried out either by first synthesizing oligonucleotide and then attaching it with solid support or by directly synthesizing the oligonucleotide over the support and removing the protecting groups from the attached oligonucleotide. Several methods for covalent attachment of oligonucleotide to solid supports have been developed (8-14). Some of these used complicated chemistry (8,11) or may leads to high levels of nonspecific adsorption of oligonucleotide or probe material(12,13). Kremsky (11) described a method for the immobilization of synthetic oligonucleotides to the latex microspheree. The oligonucleotide was modified at its 5'-end , with an aldehyde group or carboxyl group then reacted with microspheres containing hydrazide residues. The aldehyde group was introduced in four steps by first introducing the carboxylic acid linker arm at 5'-end of the oligonucleotide. The attachment of the oligonucleotides to the support through the bases as well as the ends (9) interferes with hybridization reaction. Only the method of Zhang et.al.(10) seems straight forward although it requires the derivatization of oligonucleotide with a chemical that is not yet commercially available. A method for immobilizing oligonucleotide in gel matrix attached to glass has been reported (14). Recently a

method for directly synthesizing the oligonucleotides on the support to be used for hybridization , obviating the need of deattaching them from the matrix on which they were synthesized and reattaching them to the hybridization support has been reported (15) . However, only 20-25% of oligonucleotide remained on to the hybridization support after 5 hrs of ammonia treatment at 55°C this leads to the deattachment of more than 75% of the synthesized oligonucleotide. Here, we report synthesis , linker stability and hybridization properties of disulfide and urethane linkage containing polymer supports. Our studies showed that urethane linkage containing supports retain highest percentage of oligonucleotide for hybridization even more than the ether linkage developed by Sourthen et.al. The simple hybridization experiments with the supports linked oligonucleotide revealed the hybridization yield in the range of 10-15% . However, triple helix hybridization yields were 6-9 fold of the simple hybridization yield.

MATERIALS AND METHODS

1.Synthesis of Supports

A.Synthesis of Universal Support Containing a Disulfide linkage

The synthesis of polymer supports were carried out as described earlier (16). The supports derivatized by this method and their loading capicities (18) were ,CPG-500° A, 21.0 μM/g and CPG-3000°A ,6.0 μM/g.

B. Synthesis of Support Containing Urethane Linkage

The derivatization of the support was carried out as described earlier (19) and detail procedure for its synthesis have been described else where (21). The supports derivatized by this method and their loading capacities were CPG-(500°A);

41.0 μM/gm , CPG (3000°A); 5.8 μM/g and polystyrene monobead,38.0 μM/g. Polystyrene was derivatized by DMTr dC (Bz) inplace of 3-amino-1-dimethoxytrityloxypropane and toluenediisocynate was used in place of hexamethylenediisocynate.

2.TEST OF LINKAGE STABILITY

Before Synthesis of Oligonucleotides

The linkage stability was determined by treating the derivatized support with 1 ml of 25% ammonia solution for different length of time. After fix intervals of time the ammonia solution was decanted off and dried. The polymer support was dried by adding and decanting methanol. Finally support was dried at 60° C for 1 hr in a reacti therm. The residue of cleaved linker and dried support was treated with 1 ml of perchloric acid (58 ml, 70% $HClO_4$ in 42 ml methanol) (18). The obsorbance of liberated dimethoxytrityl cation was measured at 498 nm. The stability was then determined as trityl remaining on support/trityl remaining on support + trityl on supernatant. The linkage stability of various supports are tabulated in table -1.

B. After Synthesis of Oligonucletides

Short oligonucleotides ($d(A)_4$ or $d(T)_4$) were synthesized on to the supports and the linkage stability was determined as described above . The linkage stability after the synthesis of short oligonucleotide is described in Table-2. The linkage stability after the synthesis of the oligonucleotide was also determined at room temperature by keeping the support for 24 hr in ammonia solution at room temperature. A comparison of the linker stability after synthesis of short oligonucleotides at 60°C and at room temperature has been described in table-3.

Table-1 :A Time course of Linker Stability On Supports

S.No	Support	Time (hrs)	Percentage Stability 1	6	10	15
1.	CPG(500 ^{o}A)-S-S-$(CH_2)_6$-ODMTr		51.0	44.0	32.6	10.0
2.	CPG(3000^{o}A)-S-S-$(CH_2)_6$-ODMTr		80.0	66.0	56.0	50.0
3.	CPG(500^{o}A)Urethane$(CH_2)_3$ODMTr		90.0	85.0	75.0	57.1
4.	CPG(3000^{o} A)Urethane$(CH_2)_3$ODMTr		93.7	88.6	79.7	71.4
5.	Polystyrene Urethane DMTr-dC(Bz)		68.2	55.0	60.0	25.0

Table-2: Time Course Of Linker Stability After Synthesis of Short Oligonucleotides

S.No	Support (Time in hrs)	% Linker Stability 1	6	10	15
1.	CPG(500^{o}A)-S-S-(CH_2)-ODMTr	28.0	15.6	14.4	09.0
2.	CPG(3000^{o}A)S-S-(CH_2)-ODMTr	26.6	18.7	15.0	13.7
3.	CPG(500^{o}A)Urethane$(CH_2)_3$-ODMTr	63.8	54.0	49.7	39.5
4.	CPG(3000^{o}A)Urethane$(CH_2)_3$-ODMTr	47.4	40.2	32.2	22.4
5.	Polystyrene Urethane DMTrdC-(Bz)	59.3	60.2	61.1	39.0
6.	CPG (Scurthen et. al.)*	45.0	19.0#	11.0	07.0
7.	Balbtini(Sourthen et.al.)*	37.0	28.0#	23.0	25.0

* 30-35% NH_3 at 55^{o}C was used and the loading capicities of the ether linkage were 50-60 μM/g. # % Linker stability after 5 hrs.

Table-3: Comparison of Linker Stability

S.No.	Support	% Stability		
		At 60°C for 6 hrs	At 60°C for 1hrs	At Room Temp. 24 hrs
1.	CPG(500°A)-S-S-$(CH_2)_6$-ODMTr	15.6	28.3	18.9
2.	CPG(3000°A)-S-S-$(CH_2)_6$-ODMTr	18.7	26.6	18.1
3.	CPG(500°A)Urethane-$(CH_2)_3$-ODMTr	54.0	63.8	46.0
4.	CPG(3000°A)Urethane-$(CH_2)_3$-ODMTr	40.2	47.4	35.0
5.	PolyStyrene-Urethane-DMTr-dC(Bz)	60.2	59.1	65.6

Conclusions on Linker Stability

The stability of both the linkages reduce after the synthesis of oligonucleotides on to the support. The marked reduction in stability of all the linkages was observed after the synthesis of oligonucleotides in case of control pore glass. The stability of the urethane linkage is more than the stability of disulfide linkage. The comparison of our data with the Sourthen et.al. data suggest that stability of ether linker developed by Sourthen et.al. is little more than disulfide linkages and much less than urethane linkage. A comparsion of the linkage stability at room temperature and at 60° C suggests that the ammonia treatment can be carried out at room teperature (24 hrs) for the oligonucleotide synthesised by modified phosphoramidites (PAC- Pharmacia , FOD-ABS) instead of 1 hr at 55-60 ° C for cleaving the protecting groups. The urethane linkage in both the cases seem to be promising for further studies.

3.HYBRIDIZATION STUDIES

a. Simple Hybridization Experiments

A oligonucleotide DMTrO d(CCTCTCCTCCCTTTTT), PY-16, a 16 mer was synthesized over all the five supports at 0.2 μM using standard β-cyanoethyl phosphoramidite (base protection, Bz for dA &dC ,Ibu for dG). The protecting groups were removed by giving 25% ammonia treatment at 60°C for 6 hrs. The ammonia solution was decanted off and supports were washed several times with water to remove any deprotected oligonucleotide left with support. Finally support were washed with methanol and dried. A complementary sequences d(AAAAAGGGAGGAGAGG), CPY-16 was synthesized at 1.3 μM scale over standard succinate linkage support using phosphoramidite chemistry. The oligonucleotide thus obtained by ammonia treatment was purified by 12% PAGE containing 7M urea. The CPY-16 was phosphorylated by γ^{32}P ATP using T 4 polynucleotide kinase and purified by passing through a G-15 Sephadex column. The pure ^{32}P labeled (3,60,000 cpm) CPY-16 was dissolved in 600 μl , 0.1 NaCl solution. The 100 μl containing 60,000 cpm of CPY-16 was added to each polymer support (Polymer Support Linked -PY-16,0.03 μM, 30 mg) in a microcentrifuge tube. The hybridization was allowed to procceed at 30° C for one hr. Then the microcentrifuge tube was taken out and unhybridized CPY-16 was washed with 0.1 M NaCl (5X 500 μL). The washing was done very carefully and no support particle came out with the solution while pipeting. Again 100 μL of 0.1 M NaCL was added to all the microcentrifuge tube (Containing Support Linked -PY-16 and CPY-16 hybride) and incubation was carried out at 50°C for one hr. The hybridized CPY-16, eluted out was quantitated. The quantitation of CPY-16 (unhybridized and hybridized) revealed that hybridization yield were 10.5%, 14.4%,12.6%,13.7% and 15.5% for polystyreneurethane, CPG-(500°A) urethane, CPG-(3000°A) urethane, CPG-(500°A) disulphide and CPG-(3000°A) disulphide respectively.

b. Triple Helix Hybridization Experiments

A oligonucleotide , 32 mer which forms a loop and stable duplex on annealing d(CTGGAGAGGAGGGATTTTTCCCTCCTCTCCAG) was synthesized and subsequently purified by PAGE containing 7M urea. The PY-16,oligonucleotide previously synthesized over supports forms a triple helix structure with loop duplex 32 mer at 4.5-6.5 pH (20). The 32 mer was phosphorylated by γ^{32}P ATP using T 4 polynucleotide kinase and purified by passing through a G-25 Sephadex column.

The pure ^{32}P labeled (4,20,000 cpm) 32 mer was dissolved in 300 μl (2 M NaCL, 0.2 M Sodium Acetate, Acetic Acid, pH =4.5) buffer A and annealed. The 50 μl (70,000 cpm) of annealed oligonucleotide was hybridized with the support linked oligonucleotide (0.03 μM,30 mg) at 50^{o} C for 2 hrs. After 2 hrs the unhybridized oligonucleotide was washed with buffer A (3X 300 μL). The hybridized 32 mer was unhybridized by incubated the hybrid at 50^{o}C for 20 min with 100 μl (1.0 M Tris-Cl,0.5 mM EDTA ,pH =9) buffer B. After incubation the support was washed carefully with buffer B (3X300 μl). The washings of buffer A and buffer B were quantitated. The % hybridization yields determined from these quantities were 92.4%, 88.2%, 72.5 %, 86.4% and 78.7% for polystyrene urethane, CPG(500^{o}A) urethane, CPG(3000^{o}A) urethane, CPG (500^{o}A) disulphide and CPG(3000^{o}A) respectively.

Discussion

The methods used for the synthesis of support were simple and involve well established chemistries (16,19,21). The capping was carried out in both the cases as described earlier. The synthesis of oligonucleotide was usual as was observed , when succinate linkage polymer support was used. No increase or decrease in yield during the synthesis was observed as was observed by Southern et. al. while synthesizing the oligonucleotide over newly developed ether linkage containing

supports. As an extra advantage the disulfide containing support gave a 3'-thiol oligonucleotide and urethane linkage support gave 3'-NH_2 group containing oligonucleotide on ammonia treatment. The -SH on or -NH_2 on oligonucleotides can be used for labeling reporter molecules. The linkage stability of both the linkages before the synthesis of oligonucleotide was much more than after the synthesis of oligonucleotide because linker remained buried inside the support during ammonia treatment. The tremendous decrease in stability of the linkage after oligonucleotide synthesis appears to be due to the direct exposure of the linkage with the ammonia during ammonia treatment. After 6 hrs of ammonia treatment about 16-18% of oligonucleotide remained linked with the support in case of disulphide linkage and about 40-60% remained attached with support in case of urethane linkage i.e. the urethane linkage is about two- three times more stable than disulphide linkage support. The urethane linkage was even more stable than the ether linkage developed by Sourthen et.al (Table-2). The high hybridization yield of triplex formation suggests high sequence specificity of the triple helix formation and freely availibility of the oligonucleotide linked to support for triplex hybridization. The simple hybridization yield was as accepted however quantitative (6-9 fold of simple hybridization) hybridization yield in case of triplex hybridization was interesting and suggests that development of DNA probes at the level of triplex would be advantageous.

REFERENCES

1.Molecular Cloning-A Laboratory Manual,By Maniatis,T.; Fritsch,F.;Sambrook,J.(1982) Cold Spring Harbor Laboratory.

2.Southern,E.M. (1975) J. Mol.Biol.98 , 503-517.

3.Grunstein , M. and Hogness, D.S. (1975) Proc.Nalt. Acad. Sci 72, 3961-3965.

4.Meinkoth, J.and Wahl,G. (1984) Anal. Biochem 138 ,267-284.

5.Gingeras, T.G.;Kwoh,D.V.,and Davis,G.R. (1987) Nucl. Acids Res.15,5373-5383.

6.Dunn, A.R. and Hassell, J.A. (1977) Cell 12 ,23-26.

7.Ranki,M.;Palva,A.;Virtanen,M.;Laaksonen,M. and Soderlund,H. (1983),Gene 21,77-85.

8.Ness,A.V.;Kalbfleisch,S.;Petrie,C.R.;Reed,M.W.;Tabone,J.C.and Vermenles,N.M.J. (1991) Nucl. Acids Res. 19, 3345-3350.

9.Saiki,R.K.;Walsh,P.S.;Levenson,C.H. and Erlich,H.A. (1989) Proc.Natl. Acid . Sci .USA 86 ,6230-6234.

10.Zhang,Y.;Coyne,M.Y.;Will,S.G.;Levenson,C.H.and Kawasaki,E.S. (1991) Nucl. Acids Res. 19 , 3929-3933.

11.Kremsky,J.N.;Wooter,J.L.;Dougherty,J.P.; Meyers ,R.E.;Collins ,M.and Brown, E.L. (1987) Nucl. Acids Res.15,2891-2909.

12.Ghosh,S.S. and Musso,G.F. (1987) Nucleic Acids Res.15 ,5353-5372.

13.Gingeras,T.R.;Kwoh,D.V.and Davis, G.R. (1987) Nucl. Acids Res.15 ,5373-5390.

14.Khrapko,K.R.;Lysov,Vu.P.;Khorlin,A.A.;Ivanov,I.B.;Vershov ,G,M.,Vasilenko,S.K.;Florentien,V.L.and Mirzabekov, A.D. (1991) DNA Sequence 1,375-388.

15.Maskos, U. and Southern,E.M. (1992) Nucleic Acids Res.20 ,1679-1684.

16.Kumar, A. (1993) Nucleosides and Nucleotides 12, 729-736.

17.Ellman, G.L. (1959) Arch.Biochem.Biophys.82,70-77.

18.Gait,M.J.(1984) Oligonicleotide Sytnthesis, a Practical Approach IRL Press Ltd. Oxford.

19.Sproat,B.S.and Brown, D.M.(1985) Nucl.Acids Res.13, 2978-2887.

20.Robert,R.W. and Crothers,D.M. (1991) Proc. Nalt. Acad. Sci. 88 ,9397-9401.

21.Kumar, A.; A Simple Method for Release of 3'-Aminoalkyl Oligodeoxyribonucleotides From Solid Support, Submitted , Nucleosides and Nucleotides.

Received December 21, 1993
Accepted July 13, 1994