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Oligonucleotide hybridisations on glass supports: a novel linker for oligonucleotide synthesis and hybridisation properties of oligonucleotides synthesised *in situ*

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ABSTRACT

A novel linker for the synthesis of oligonucleotides on a glass support is described. Oligonucleotides synthesised on the support remain tethered to the support after ammonia treatment and are shown to take part in sequence specific hybridisation reactions. These hybridisations were carried out with oligonucleotides synthesised on 'ballotini' solid sphere glass beads and microscope slides. The linker has a hexaethylene glycol spacer, bound to the glass via a glycidoxypropyl silane, terminating in a primary hydroxyl group that serves as starting point for automated or manual oligonucleotide synthesis.

INTRODUCTION

The rapid progress in molecular biology has depended on methods of detecting specific DNA or RNA sequences by molecular hybridisation, reviewed in (1). For the detection of mutations and polymorphisms for which the nucleotide sequence is known, Wallace and coworkers (2) introduced the differential hybridisation of oligodeoxyribonucleotide probes. The main advantage of oligonucleotide hybridisation lies in the possibility to detect single nucleotide changes, not feasible with longer probes that are less sensitive to mismatching.

For some applications DNA to be analysed is immobilised on nitrocellulose filters or nylon membranes and probed with labelled oligonucleotides (3). For other applications, e.g. isolation of mRNA (4) or DNA diagnostic purposes (5, 6), oligonucleotides are immobilised on solid supports and used to capture the macromolecule of interest. A number of methods have been reported for immobilising oligonucleotides on supports (5–11). Some of these use complicated chemistry (7, 8) or may lead to high levels of unspecific adsorption of oligonucleotide or probe material (9, 10). Furthermore, attachment through the bases as well as the ends (5) interferes with the hybridisation reaction. Only the method of Zhang *et al.* (6) seems straightforward although it requires the derivatisation of oligonucleotides with a chemical that is not yet commercially available. A method for immobilising oligonucleotides in a gel matrix attached to glass has recently been reported (11).

Solid phase methods for synthesising oligonucleotides offer the opportunity to make oligonucleotides directly on the support to be used for the hybridisation, obviating the need of detaching them from the matrix on which they were synthesised and re-attaching them to the hybridisation support.

The linker employed in standard oligonucleotide synthesis (12) is labile in the conc. ammonia used for the deprotection of the bases. The linker described here uses only the most stable bonds known in organic chemistry, viz. carbon–carbon, carbon–silicon, and ether bonds; it requires only readily available chemicals, is easy to synthesise and stable in the ammonia deprotection step.

MATERIALS AND METHODS

Linker synthesis

3-Glycidoxypropyltrimethoxysilane, hexaethylene glycol, pentaethylene glycol, ethylene glycol and HPLC grade acetonitrile were purchased from Aldrich and used without further purification. Underivatised CPG (controlled pore size glass, pore size 500Å, 37–74 μm particle size) was purchased from Pierce, ballotini glass beads (Grade 18, diameter range 40–75 μm, and Grade 13, diameter range 90–135 μm) were from Jencons. All DNA synthesis materials were purchased from Applied Biosystems.

In typical experiments, underivatised CPG (10 g) or ballotini glass beads (20 g) were suspended in a mixture of xylene (40 ml), 3-glycidoxypropyltrimethoxysilane (12 ml) (modified after [13]) and a trace of Hünig's base (after [14]) at 80°C overnight with stirring, then washed thoroughly with methanol, ether, air dried and dried *in vacuo*. Alternatively, beads were suspended in a solution of 5% of the silane in water, keeping the pH between 5.5 and 5.8 (after [15]). Reaction was for 30 minutes at 90°C. An alternative 'vapour deposition' strategy of derivatisation has also been reported (16).

In a second step these beads were heated with stirring in neat diol (hexaethylene glycol, pentaethylene glycol or ethylene glycol), containing a catalytic amount of conc. sulphuric acid, overnight at 80°C to yield alkyl hydroxyl derivatised support. After washing with methanol and ether the beads were air dried, then dried *in vacuo* and stored under Argon at −20°C. In addition to reaction with diol, a dilute solution of HCl in water was used to cleave the epoxide residue to yield a primary hydroxyl group (Figure 2). Alternatively, 40 ml of anhydrous dioxane containing 32 mmol of the alcohol and 1 ml boron trifluoride etherate were added and reaction carried out for 30 min at 90°C.

Microscope slides (BDH Super Premium, 76×26×1 mm) were derivatised in the same way. Reaction was carried out in a staining jar fitted with a drying tube. The slides were washed with methanol, ether, air dried and stored desiccated at −20°C until use.

Automated synthesis of oligonucleotides

Standard programmes were used for all oligonucleotide syntheses on an Applied Biosystems 381A automated synthesiser. Trityl effluents were collected, diluted to 10 ml with 0.1 M toluenesulfonic acid (Aldrich) in acetonitrile and absorbance measured in a 1 cm quartz cuvette on a Bausch & Lomb Spectronic 2000 spectrophotometer.

Manual synthesis of oligonucleotides

For the manual synthesis of oligonucleotides on a microscope slide, the synthesis cycle was performed as follows: The coupling solution was made up fresh for each step by mixing six volumes of 0.5 M tetrazole in anhydrous acetonitrile with five volumes of a 0.2 M solution of the required β-cyanoethylphosphoramidite (12). Coupling time was three minutes. Oxidation with a 0.1 M solution of iodine in tetrahydrofuran/pyridine/H_2O yielded a stable phosphotriester bond. Detritylation of the 5′ end with 3% trichloroacetic acid in dichloromethane allowed further extension of the oligonucleotide chain. There was no capping step since the excess of phosphoramidites used over reactive sites on the slide was supposed to be large enough to drive the coupling to completion.

As the chemicals used in the coupling step are moisture-sensitive, this critical step must be performed under anhydrous conditions in a sealed container: The shape of the patch to be synthesised was cut out of a sheet of silicone rubber (76 ×26×0.5 mm) which was sandwiched between a derivatised microscope slide and a piece of teflon of the same size and thickness, to which was fitted a short piece of plastic tubing that allowed injection and withdrawal of the coupling solution by syringe, and flushing with argon. The assembly was held together by fold-back paper clips. After coupling the set-up was disassembled and the slide put through subsequent chemical reactions (oxidation with iodine, and detritylation by treatment with trichloroacetic acid) by dipping it into staining jars.

After the synthesis, the oligonucleotides were deprotected by putting the slide into a Schott bottle containing 30% NH_3 and incubating for 5 to 10 hours at 55°C in a water bath.

Set-up for column chromatography

A glass capillary (diameter 1.0 mm) was drawn out at one end to a narrow opening which was plugged with crushed glass particles and sintered in a flame. The inside of the capillary was silanised, approximately 40 mg of derivatised ballotini glass beads were layered on the glass frit and the top of the column connected to a syringe driven by an infusion pump. Hybridisation solution and washing solutions were applied to the column at rates in the range of 3−10 μl/min.

RESULTS AND DISCUSSION

Linker synthesis

The initial derivatisation of the solid support is carried out in two steps: The first reaction is a condensation of 3-glycidoxypropyltrimethoxysilane to the solid support bearing 'silanol' groups (Figure 1a). When 'wet' solvent is used or the

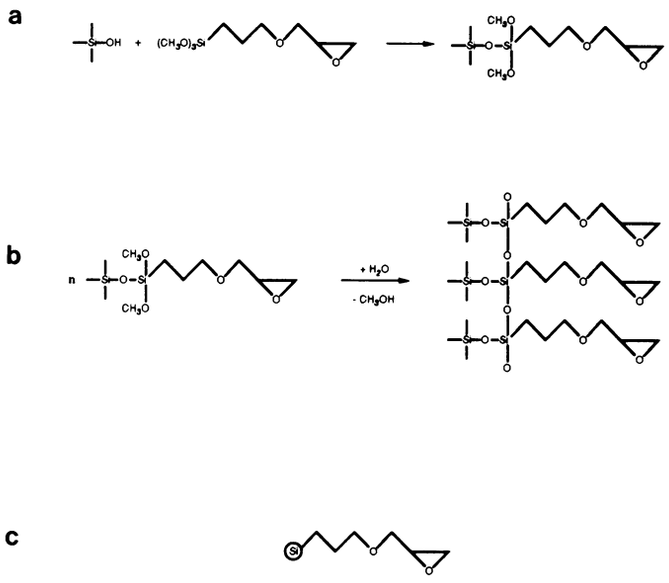

Figure 1. The first step in the derivatisation.

reaction is carried out in water, further crosslinking occurs (17), cf. Figure 1b, which can be abbreviated as in Figure 1c. In the second step the epoxide group is cleaved with a diol or water under acidic conditions. Several compounds were used to yield linker molecules with different chain lengths, cf. Figure 2.

Determination of loadings. The loadings achieved on CPG, ballotini beads and the slide were determined by carrying out standard oligonucleotide synthesis using both phosphoramidite and Hydrogen-phosphonate chemistry on an Applied Biosystems Model 381 A with derivatised CPG or ballotini beads in the column. Coupling yields were measured from the detritylation effluent using a standard procedure (18).

The first coupling step gave a high trityl yield, ca. 3−3.5 times that obtained with the commercially available LCAA-CPG support (Table I). Stepwise yields fell to between 85 and 95% for cycles 2 and 3, but in later steps coupling yields rose to close to 100%. The high first coupling could indicate that some diol is only loosely bound and removed during the synthesis cycles.

To test that the epoxide is still intact after attachment, epoxide-derivatised CPG was analysed in the same way. In this case, the coupling yields *increased* in the course of synthesis presumably because cleavage of the epoxide by the chemicals used in the reactions exposes hydroxy alkyl groups.

The properties of derivatised supports (Table I) show that the loadings per unit weight for ballotini beads (solid spheres) is only a fraction of that for porous glass, as expected from its smaller surface area. The yields for ethylene glycol, pentaethylene glycol and hexaethylene glycol derivatised CPG are very similar, whereas HCl/H_2O treated epoxide-support shows a lower loading consistent with the notion of increased steric interference with a short linker. The epoxide alone gives a yield only slightly lower than untreated CPG (which is presumably due to reaction with surface silanol groups). This small amount could be due to epoxide cleaved in the derivatisation or by the chemicals used in oligonucleotide synthesis or to incomplete derivatisation of the whole surface (some silanol groups remain unreacted). It is also an indication that most of the epoxide remains intact after the

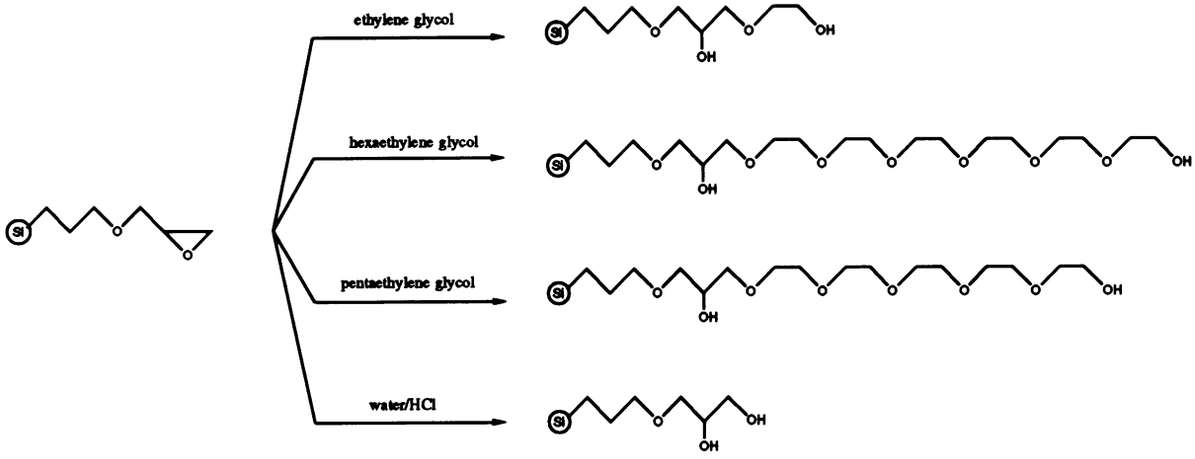

Figure 2. The second step in the derivatisation.

Table I. Loadings of linker molecules on glass supports

Molecules used for the derivatisation	Support	Loading nmol/mg	After ammonia treatment	Percentage remaining
CPG only	CPG	24.9	–	–
epoxide only	CPG	17.4	43.5	n/a
epoxide/HCl/H_2O	CPG	44.8	41.1	81
epoxide/ethylene glycol	CPG	48.9	47.5	94
epoxide/pentaethylene glycol	CPG	53.3	46.2	75
epoxide/hexaethylene glycol	CPG	50.8	45.4	81
commercial LCAA epoxide/hexaethylene glycol	CPG	14.2	–	–
	Ballotini 90–135 μm	0.07	–	–
epoxide/hexaethylene glycol	Ballotini 40–75 μm	0.05	–	–

Oligonucleotide syntheses were performed with approx. 4 mg CPG (or 120 mg ballotini) before and after ammonia treatment of the support and the detritylation effluent collected. Since cleavage is expected to produce silanol groups which will also take part in a coupling reaction coupling yields measured in this way are the sum of the reaction of these silanol and of linker hydroxy alkyl groups, given by the formula

$$OD_m = (1 - x)OD_a + xOD_s$$

with: x amount of cleavage; OD_m optical density measured at 498 nm per mg after deprotection; OD_a optical density of effluent from alkyl derivatised support before deprotection; OD_s optical density of effluent from silanol groups of underivatised CPG

The equation can be solved to give

$$x = \frac{OD_a - OD_m}{OD_a - OD_s}$$

The percentage remaining is then defined as $1 - x$, and results have been included in the table. The value for the third coupling steps was used.

initial condensation as proven by the much higher loading obtained after ammonia treatment.

Tests of linker stability. We have found that the linker is not completely stable to ammonia. Stability was determined in two different ways.

In a first series of experiments, the hexaethylene glycol derivatised beads were transferred into screw-capped microcentrifuge tubes after 'trityl-on' oligonucleotide synthesis (short runs of T or C) and treated with conc. ammonia at 55°C for different lengths of time. To test the linker stability, the amount of dimethoxytrityl groups in the ammonia supernatant and remaining on the support was determined as described in the Table II legend.

Table II. A time course of linker stability on glass supports.

Incubation time (hrs)	1	5	10	15
Stability on CPG (%)	45	19	11	7
Stability on ballotini (%)	37	28	23	25

Short oligonucleotides bearing a 5'-dimethoxytrityl group were synthesised on CPG (ca. 2 mg) or ballotini (ca. 90 mg) supports that were subsequently treated with conc. ammonia at 55°C for the times indicated. The supernatant was removed, the beads rinsed several times with conc. ammonia, the supernatants combined and evaporated. The residue of cleaved linker and the dried supports were treated with 80% acetic acid for 30 min to hydrolyse the dimethoxytrityl group and evaporated. 600 μl methanolic $HClO_4$ (60 ml 60% $HClO_4$ in 37 ml methanol) was added and the liberated dimethoxytrityl cation measured from the absorption at 498 nm. Stability is then defined as (trityl remaining on beads) / (trityl remaining on beads and in supernatant).

Control measurements were carried out on plain CPG (0.4% remaining after 5 hrs), standard ABI nucleoside derivatised support (0.8% after 1 hr), and LCAA–CPG (26% after 1 hr). 90% remain with the new linker after 30 min ammonia treatment at r.t.

As an independent measure of linker stability, and to detect any diol adsorbed to the surface after derivatisation or extensive polymerisation (15, 17), an aliquot (~50 mg) of the linker derivatised CPG was put through a 16 hr ammonia deprotection to remove adsorbed diol and cleave any linker molecules labile to ammonia. Oligonucleotide synthesis on the treated supports showed slightly lower first coupling yields as compared with untreated support, with subsequent stepwise yields of 96–100%. Comparing the coupling values to those measured before ammonia treatment (the value for the third coupling was used) gives an indirect estimate of the linker stability as detailed in the caption to Table I. For the epoxide the measured loading rose 2.5 times after ammonia treatment because epoxides are hydrolysed under these conditions to yield glyceroloxypropyl-CPG (Figure 2), which serves as a substrate for oligonucleotide synthesis. The values for mono-, penta-, and hexaethylene glycol derivatised support are similar, which would indicate a high stability of the linkage.

Table III. A comparison of properties of different supports

Support	CPG	Ballotini 90–135 μ	Ballotini 40–75 μ	Microscope slide
Surface area	70 m^2/g	200 cm^2/g	350 cm^2/g	1300 mm^2
Highest loading (pmol/mm^2)	1.1	5.2	2.4	1.08
Average loading (pmol/mm^2)	0.6	3.3	1.5	0.39
Stability (5 hr incubation)	19%	28%	28%	(28%)
Loading after deprotection (pmol/mm^2)	0.11	0.92	0.42	0.11
Surface area per molecule ($Å^2$)	38.2^2	13.4^2	19.9^2	39.1^2

Table IV. Effect of oligonucleotide concentration on hybridisation rate

Relative concentration	50	20	10	2	1
% of input hybridised	23	14	5.5	0.8	0.6
Relative rate	38	23	9	1.5	1

Hybridisation for 2 hrs at 30°C in 0.1 M NaCl in 1.5 ml centrifuge tubes was followed by removal of the supernatant, three washes with 0.1 M NaCl at 0°C and determination of the amount of radioactivity associated with the beads by Čerenkov counting.

Table V. Effect of temperature on elution rate

Elution time 5′	20′	50′	110′	230′	
Temperature	% eluted				% remaining
30°	23	38	54	71	85	0
36°	34	61	78	90	96	3
44°	42	54	67	87	97	2
65°	74	89	96	98	98	1.4

To each of five tubes was added an approximately equal number of beads and complementary oligonucleotide (30,000 c.p.m.) in 50 μl 0.1 M NaCl. Hybridisation was carried out at 30° overnight to maximise the amount of hybrid. The solution was removed and the beads washed twice with ice-cold 0.1 M NaCl. Elution was for increasingly longer times at a different temperature for each tube. After each interval the supernatant was removed, the beads were washed twice with ice-cold NaCl solution (100 μl), and eluted with prewarmed NaCl solution (100 μl). Elution times are cumulative times, i.e. the last time point was taken 230 min (~4 hrs) after the beginning, and 2 hrs after the previous one. The four temperatures indicated were kept constant for the four tubes. The values in the five columns denote the cumulative amount of material that had been eluted at the indicated times as determined by scintillation counting and listed as the fraction of total material bound. The last column gives the percentage of counts that remained after an elution in TE at 60°C (for the 30°C elution), or after 4 hrs of elution at the indicated temperatures, respectively.

The values obtained in the two assays differ considerably (Tables I and II). However, the two assays are both indirect and experimental error could account for the differences. As will be seen, the amount of oligonucleotide remaining is sufficient to provide sensitive detection of hybridisation.

The stability on ballotini beads is higher than on CPG, possibly because the latter consists of almost pure silica (19), which might partially dissolve under these conditions, thus accounting for part of the instability. The other most likely point of instability is the attachment site of the silane to the glass, which is only a single silicon-oxygen-silicon bond if cross-linking is incomplete (see Figure 1a and b) or if polymerisation occurs (17).

Loading on a microscope slide. Oligonucleotide synthesis was carried out over the whole surface of a microscope slide and the detritylation effluent collected and quantified (Table III). The loadings per unit area on CPG and the microscope slide correspond well, whereas the ballotini loading is higher by a factor of approx. 4–8. This could be a sign of more successful derivatisation of ballotini beads since the value of one molecule per $(13.4\ Å)^2$ is close to the $(5\ Å)^2$ value for silanol groups in porous glass (14). (The value before ammonia treatment is $(5.7\ Å)^2$.) The diffusion of reactants into the pores of CPG might be impeded by narrow pore size, and the derivatisation of microscope slides without stirring could lead to lower yields.

Hybridisation to tethered oligonucleotides

The linker was developed to provide a convenient way of making support bound oligonucleotides to use in nucleic acid hybridisation. The behaviour of the supports in hybridisation was explored using ballotini glass beads.

To permit the use of small numbers of beads, which would be difficult to handle in a column or centrifuge tube, beads were immobilised by fusing them to plastic cocktail sticks (2 cm long) which were dipped to ca. 1 mm in molten polypropylene, then brought into contact with a pile of derivatised glass beads and allowed to cool. Approximately 100–200 beads adhered to each stick. A stick with approx. 100 glass beads derivatised with the sequence 3′-AGGTCGCCGCCC was dipped into 30 μl of a solution containing the complementary oligonucleotide 5′-TCC-AGCGGCGGG (30,000 c.p.m., 80 fmol in 30 μl of 0.1 M NaCl, 37°C). After hybridisation for various times and at different temperatures the stick was removed from the tube, rinsed, and the bound material eluted by dipping the stick into 0.1 M NaCl at 50°C. Typical hybridisation yields were: 4% at 37°C for 30 min; 5.3% at 30°C for 55 min; 13% at 30°C for 16 hrs. Nonspecific binding was between 0.05% and 0.2% of input counts. Under the same conditions (16 hrs at 30°C) the non-complementary oligonucleotide 5′-GGGCGGCGACCT showed only 0.2% binding of input counts.

Table VI. Properties of derivatised ballotini beads

Bead size (μm)	90–130	40–75
Surface are per bead (mm^2)	0.038	0.011
Volume per bead (mm^3)	6.97×10^{-4}	1.13×10^{-4}
Values calculated for radius (μm)	55	30
Density (g/cm^3)	2.95	2.95
Mass per bead (ng)	2.06	0.33
Number of beads per mg	486	3000
Surface area per mg (mm^2)	18.5	33.0
Oligonucleotide loading per mg (pmol)		
highest yields	~104	~81
average yields	~66	~51
yield after deprotection	~18	~14
Oligonucleotide loading per bead (fmol)	38	4.8

Hybridisation and elution behaviour. For larger scale work hybridisations with beads—typically 1 mg—were carried out in 0.5 ml centrifuge tubes. The dependence of rates of hybridisation and elution on concentration and temperature were determined (Tables IV and V).

The concentration of input oligonucleotide over a 50-fold range showed an almost proportional change in yield of hybrid (i.e. rate of hybridisation, Table IV, compare 'relative concentration' to 'relative rate'), compatible with the expected pseudo first order reaction kinetics.

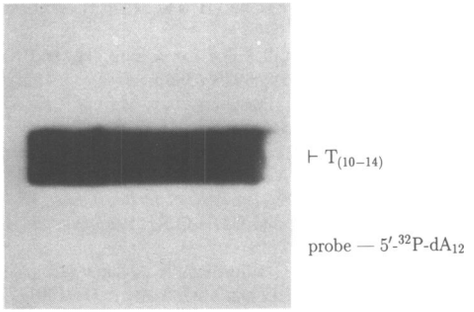

Figure 3. Hybridisation to a slide bearing sequences $T_{10}-T_{14}$. 10 pmol oligo-A_{12}, labelled to a total activity of 1.5 million c.p.m. was used in a hybridisation carried out in a perspex (Plexiglass) container made to fit a microscope slide filled wit 1.2 ml of 1 M NaCl in Tris−HCl pH 7.5, containing 0.1% SDS, for 5 minutes at 20°C. After a short rinse in the same solution without oligonucleotide more than 2,000 c.p.s. could be detected with a handheld monitor.

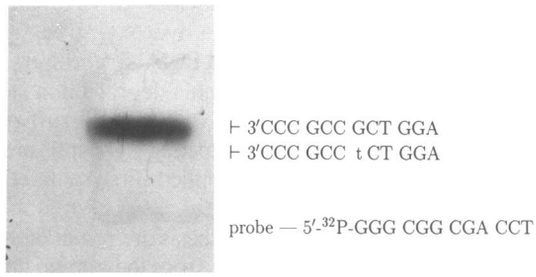

Figure 4. Hybridisation to a slide bearing *cosL* and a related sequence. The probe oligonucleotide *cosR* was kinase labelled with ^{32}P to 1.1 million c.p.m. Hybridisation was for 5 hours at 32°C in 0.1 M NaCl, Tris−HCl pH 7.5, 0.1% SDS.

The highest concentration of oligonucleotide was 160 fmol (13,000 c.p.m.) in 20 μl. Only 0.03% bound non-specifically and could not be eluted. Under the same conditions only 0.02% non-complementary oligonucleotide bound to the beads (12 out of 65,000 c.p.m.) as compared to 23% for a similar concentration of complementary oligonucleotide, a further indication that this method of isolating DNA fragments is very specific and clean, as compared with other solid phase methods (9, 10). The elution rate increased with temperature, as expected (Table V). Three times as much material eluted at 65°C as at 30°C, within 5 min. Even at 30°C, which is below T_m, there is a non-negligible rate. Little material remains after 4 hrs under all conditions of elution.

Column chromatography. Another convenient way to isolate oligonucleotides and to test the hybridisation behaviour of the novel support is by column chromatography (see MATERIALS AND METHODS). In a typical experiment using the derivatised beads described above and the same complementary oligonucleotide, 0.2 pmol of labelled oligonucleotide (34,000 c.p.m.) in 1 ml of a solution of 0.1 M NaCl was applied to the column at a rate of 3 μl/min. The jacketed column was kept at 35°C. 90 μl fractions were collected in microcentrifuge tubes and the flow-through monitored by Čerenkov counting. The column was washed in 0.1 M NaCl at 35°C and then 48°C. 70% of the oligonucleotide bound to the beads, of which 99.9% was eluted at the higher temperature. The high specificity of hybridisation was demonstrated by carrying out a similar experiment with oligonucleotide 5′-GGGCGGTGACCT, and though this produces a single G-T mismatch at position 7, a mismatch that is the least destabilising (20), there was only 0.5% binding at 35° and 0.2% at 40°C.

Table VI contains a short summary of the physical parameters of ballotini glass beads. These experiments suggest that the derivatised beads will be useful in the chromatographic separation of nucleic acids.

Synthesis of oligonucleotides on a microscope slide and hybridisation behaviour

The experiments with beads demonstrate desirable properties of specific and fast hybridisations. For certain applications (21, 22) it would be desirable to synthesise a number of different oligonucleotides side by side on a flat surface, e.g. a slide. This format would permit rapid and simple analysis of many reactions simultaneously using techniques well-established for filter hybridisations. After the initial synthesis of oligonucleotides on the slide, it can essentially be treated like a membrane and analysed by autoradiography or phosphorimaging (23).

A slide bearing sequences $T_{10}-T_{14}$. Oligo-T_{10} to oligo-T_{14} were synthesised on a slide by gradually decreasing the level of the coupling solution in steps 10 to 14 (see MATERIALS AND METHODS). Thus the 10mer was synthesised on the upper part of the slide, the 14mer at the bottom and the 11-, 12- and 13mers were in between. The slide was probed with A_{12}. An autoradiograph (Figure 3) showed that the probe hybridised in high yield to the area where the oligonucleotide had been synthesised, with no detectable non-specific binding to the glass or to the region that had been derivatised with the linker alone.

By gradually heating the slide in the wash solution the T_d was determined to be ca. 33°C (in 1 M NACl). No counts were detectable after incubation at 39°C. The cycle of hybridisation and melting was repeated eight times with no diminution of the signal. Around 5% of the input counts were taken up by the slide at each cycle.

Effect of a mismatch. The specificity of hybridisation seen on the columns was tested on the slide using a similar set of oligonucleotides. Two sequences, 3′-CCCGCCGCTGGA (*cosL*), complementary to the left cohesive end of bacteriophage λ, and 3′-CCCGCCtCTGGA were synthesised which differ by one base at position 7. All bases except the seventh were added in a rectangular patch as outlined in MATERIALS AND METHODS. At the seventh base, half of the rectangle was exposed in turn to add the two different bases, in two adjacent stripes. After hybridisation of *cosR* probe oligonucleotide (5′-GGGCGGCG-ACCT) 100 c.p.s. could be detected on the front of the slide after rinsing. Autoradiography showed that annealing occurred only to the part of the slide with the fully complementary oligonucleotide (Figure 4). No signal was detectable on the patch with the mismatched sequence.

CONCLUSIONS

The three requirements for a suitable linker, that it should be easy to synthesise, bearing a hydroxyl group, and be sufficiently stable to the harsh conditions used for the deprotection of the bases are satisfied by the two step synthesis described here. The chemicals required are readily available, the first step takes 30

minutes, the second can be conveniently carried out in an overnight reaction. The linker bears a hydroxyl group and can be used directly on CPG in automated DNA synthesisers. Some of the linker is removed by the ammonia deprotection step, but the oligonucleotide remaining after the treatment is sufficient for hybridisation detection. Base protecting groups are now commercially available (e.g. from Applied Biosystems) that can be removed by a very short treatment.

Initial yields of oligonucleotide synthesis are remarkably high, close to what is theoretically possible from the known chemical composition of the glass surface. The area at the glass surface occupied by each oligonucleotide is approx. $(6\ Å)^2$ to $(12\ Å)^2$. The length of the linker is ca. 45 Å, and each nucleotide residue adds ca. 20 Å to the length and considerable bulk. With such tight packing it is possible that they would interfere sterically with neighbouring oligonucleotides. Even with the losses due to ammonia treatment, the oligonucleotides are closely packed at one per $(40\ Å)^2$ on CPG and the slide that give lower loadings (Table III). The probes used in the work described here were short, comparable in length with the tethered oligonucleotide. However, in work to be described elsewhere (22), we have shown that longer sequences, in excess of 100 bases, can form duplexes with oligonucleotides down to 12mers, tethered through a 20 atom linker, with little sign of steric interference from the solid support. In general, the stabilities of tethered duplexes are similar to those of oligonucleotides in solution. The kinetics of the hybridisation agree with the expected pseudo first order reaction. Hybridisation is highly specific and sensitive to a single base mismatch. In these senses, the system appears to be homogeneous, well behaved and predictable.

Two physical forms were developed, which will find quite different applications. Beads, either of porous or conventional glass, are readily produced in automated oligonucleotide synthesis machines and can be used in bulk procedures, such as columns, to isolate specific nucleic acid sequences. Support bound DNA is also used to isolate DNA binding proteins. Most DNA binding proteins bind to short sequences of double-stranded DNA. Such sequences could readily be made as 'fold-back' sequences using the method described here, though we have not yet tested this approach.

The linkage also allows the manual synthesis of oligonucleotides on slides as a pre-requisite for constructing large arrays of oligonucleotides to be used in DNA sequence analysis and the detection of mutations, to be reported elsewhere (21, 22).

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