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A new hydrophobic linker effective for the in situ synthesis of DNA–CPG conjugates as tools for SNP analysis

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Abstract—DNA chips consisting of DNA oligonucleotide probes immobilized on the surface of solid supports are very powerful tools for rapid analysis of multiple samples. In this Letter we describe a new method for the efficient synthesis of DNA probes without their serious elimination by use of a new hydrophobic 16-hyroxydecanoic linker and a new non-aqueous reagent of $MeNH_2$/THF for the deprotection of the base and phosphate protecting groups on CPG resins. The elimination of DNA probes in this new method could be suppressed more than 20-fold compared with the previous method using a hexaethylene glycol linker and concd NH_4OH. Moreover, we carried out SNPs detection by use of our DNA–CPG conjugate to show the utility of our new linker and deprotection conditions.

Development of methods for the detection of single nucleotide polymorphisms (SNPs) has proved to be of great importance for identification of disease-causing genes and for administration of suitable drugs.[1] A variety of methods for SNPs detection have been reported to date.[2] Among them, the strategy using DNA chips consisting of DNA oligonucleotide probes immobilized on the surface of supports have proved to be useful for rapid simultaneous analysis of many samples.[3]

Recently, Tsukahara and Nagasawa have developed a new strategy called 'probe-on-carrier method' where small particles of DNA–CPG conjugates, which were synthesized by a DNA synthesizer, were arranged in specified places on a glass plate and used for SNPs detection.[4] It turned out that this conjugate has neither self-fluorescence nor non-selective interaction with proteins or oligonucleotides in cell extracts. It is also well known that the efficiency of the condensation in the current DNA synthesis on CPG is sufficiently high to give an almost single peak of the desired DNA oligomer in HPLC upon its release from this support. These properties strongly suggested that the probe-on-carrier method would be more practical and promising. Furthermore, the inherent macroporous property of CPG allows high density of DNA probes in its inner three-dimensional space so that the sensitivity of detection in such an improved method would dramatically increase compared with that of DNA probes of the conventional glass plate that can use only the 2-dimensional surface. In addition to these advantages, CPG can be freely manipulated in an appropriate form by cutting so that flat CPG plates having a uniform thickness are readily available and can be used as the support of DNA chips simply by putting them on a slide glass after the DNA synthesis on the CPG plate.

However, we have noticed a serious problem that most of the DNA probes synthesized were eliminated from CPG by treatment with ammonia required for removal

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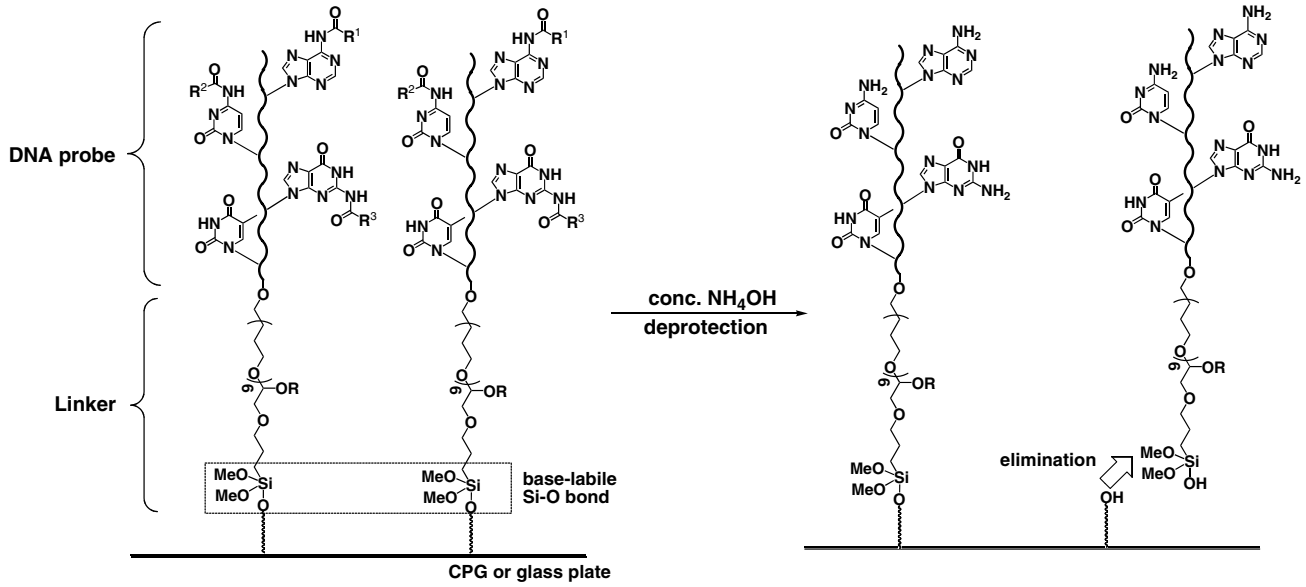


Figure 1. Elimination of DNA probes from the CPG or glass plates by treatment with concd NH_4OH required for deprotection.

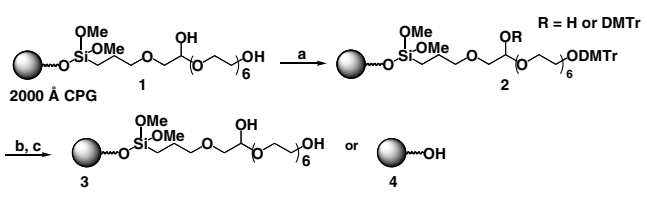
of the base and phosphate protecting groups at the final stage. Now, it turned out that this undesired elimination was caused by the inherent base-lability of the Si–O bond of Southern's linker[5] used for connection of the DNA probe to CPG, as shown in Figure 1. Similar elimination of DNA probes by treatment with NH_4OH on glass plates was also observed in Gao's study.[6]

In this Letter, we report a new linker effective for the synthesis of DNA–CPG conjugates that can suppress elimination of DNA probes from CPG, maintaining their appropriate probe-density and increasing their binding affinity for DNA. We also describe applications of these DNA–CPG conjugates to the SNP analysis by use of molecular beacon (MB).

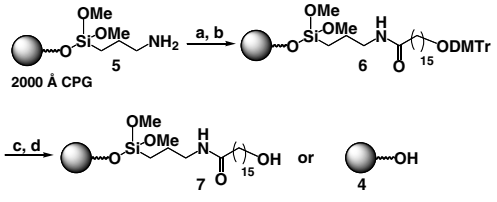
Southern et al. reported a hexaethylene glycol linker that can immobilize oligoDNA probes on slide glass supports.[5] We examined the stability of DNA probes having this hexaethylene glycol linker on the CPG resin under the deprotection conditions required for the usual DNA synthesis by using concd NH_4OH, as shown in Scheme 1. The tritylation of 2000 Å CPG **1** having a hexaethylene glycol linker with DMTrCl in pyridine gave resin **2**. The DMTr group was introduced into the CPG resin with the loading amount of 10 μmol/g. Resin **2** was subsequently treated with concd NH_4OH at room temperature for 8 h. As a result, serious cleavage of the Si–O bond between the linker and CPG occurred so that resin **2** was nearly quantitatively converted to the resin **4**, as determined by the DMTr cation assay. This result suggested that most of the DNA probes were eliminated from the CPG support. It is likely that the silicate bond is too labile during the ammonium hydroxide treatment.

Next, we introduced a hydrophobic linker into CPG to avoid the nucleophilic attack of the hydroxide ion on the Si–O bond. The desired resin **6** having a 16-hydroxyhexadecanoyl residue was synthesized by condensation of DMTrO–$(CH_2)_{15}$COOH with CPG resin **5** containing a 3-aminopropyl group, as shown in Scheme 2. It was found that the Si–O bond cleavage on resin **6** upon treatment with concd NH_4OH for 8 h was greatly suppressed to a degree of 4% and 13% at room temperature and at 55 °C, respectively, as determined by the DMTr cation assay.

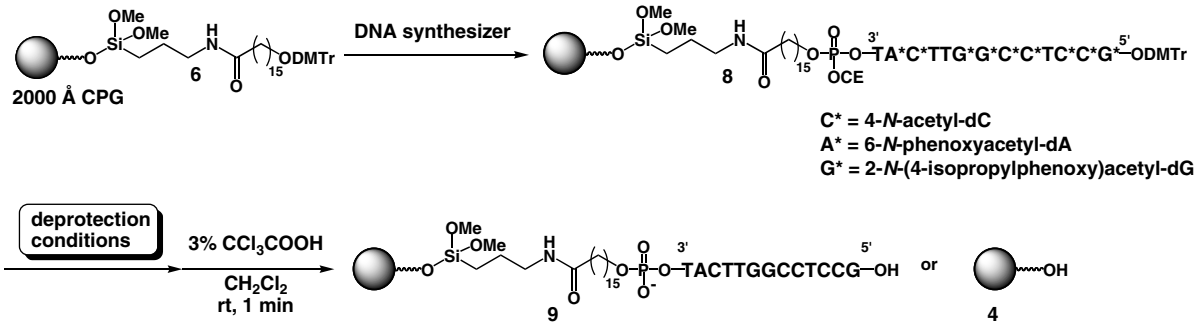
Similarly, resin **8** containing an N- and phosphate-protected oligonucleotide 13mer was treated with concd NH_4OH to examine the stability of the hydrophobic linker on the CPG, as shown in Figure 2. Unexpectedly, 98% and 85% losses of the probe DNA were observed at 55 °C and at room temperature, respectively, as shown in entries **1** and **2** of Figure 2. The elimination of the DNA probe also occurred to a degree of 45% when it was treated with 40% aqueous $MeNH_2$ at room temperature for 3 h (entry **3**). These considerable losses



Scheme 1. Reagents and conditions: (a) DMTr–Cl, pyridine, rt, 12 h; (b) concd NH_4OH, rt, 8 h; (c) 3% CCl_3COOH, CH_2Cl_2, rt, 2 min.



Scheme 2. Reagents and conditions: (a) DMTrO–$(CH_2)_{15}COO^-H^+$-NEt_3, DCC, CH_2Cl_2, rt, 12 h; (b) Ac_2O, DMAP, pyridine, rt, 2 h; (c) concd NH_4OH, rt, 8 h; (d) 3% CCl_3COOH, CH_2Cl_2, rt, 1 min.



Entry	Deprotection conditions	Ratio of elimination of DNA probes from the CPG resin[a]
1	concd NH_4OH, 55 °C, 8 h	98%
2	concd NH_4OH, rt, 8 h	85%
3	$MeNH_2$ aq, rt, 3 h	45%
4	ethylenediamine - EtOH (1:1, v/v), rt, 3 h	10%
5	2 M NH_3/MeOH, rt, 3 h	5%
6	2 M $MeNH_2$/THF, rt, 3 h	4%

[a]The ratios were estimated by using the DMTr cation assay.

Figure 2. Elimination of DNA probes from the CPG resin under the deprotection conditions.

of the DNA probe by the nucleophilic attack of the hydroxide ion might result from an increase of the hydrophilicity on the surface of the resin containing a number of phosphodiester linkages. Therefore, non-aqueous conditions were used for the selective deprotection to suppress the cleavage of the Si–O bond. The use of ethylenediamine/EtOH reported by McGall (entry **4**)[7] resulted in considerable decrease of elimination of the DNA probe to a degree of 10%. Moreover, more than 95% of the DNA probes was found to remain intact under mild non-aqueous conditions such as 2 M NH_3/MeOH and 2 M $MeNH_2$/THF[8] for 3 h (entry **5** or **6**). On the other hand, more than 5-fold elimination of the DNA probe on the CPG containing a hexaethylene glycol linker (a degree of 26%) was observed compared with a hydrophobic linker. As the result, we could for the first time carry out an efficient synthesis of DNA probes without their serious elimination by use of a new hydrophobic linker under mild non-aqueous conditions on CPG resin. These conditions might be also generally useful for the in situ synthesis of DNA probes on a glass plate.

Finally, we carried out SNPs detection by use of our CPG conjugate to show the utility of our new linker and deprotection conditions. By use of the so-called MB probe[9] having a fluorescence group and its quenching group, non-labeled complementary target DNA sequences can be detected. We synthesized MB probes (the density of this MB probe was 32 pmol/cm^2) having a p53 sequence on a CPG plate **10** and studied the SNP detection in the 947th and 966th regions of a human p53 gene[10] by use of this plate, as shown in Figure 3. The MB probe having a fluorescein (F) group and a dabsyl (Q) group was synthesized on 2000 Å CPG in more than 99% average coupling yield according to the procedure described above. In further study, we found that the $(CH_2O)_6$ spacer was a suitable hydrophilic spacer of

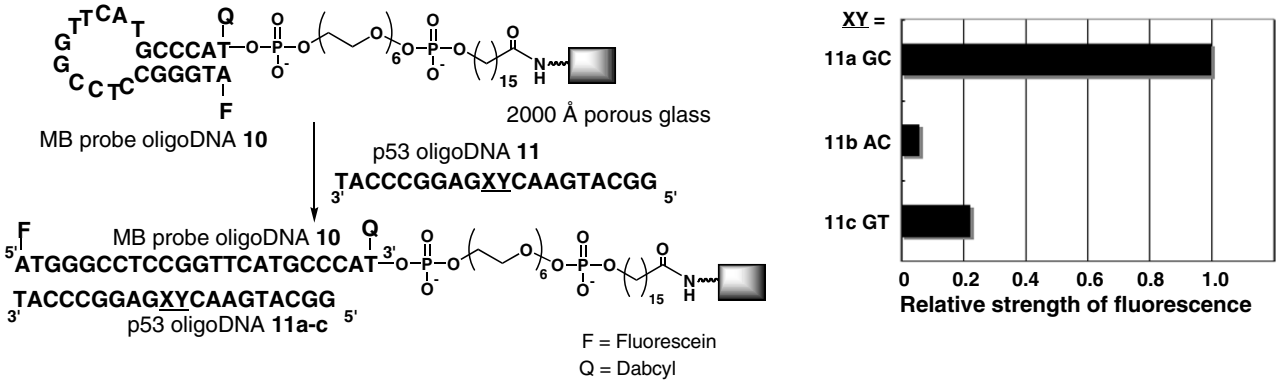


Figure 3. The match/mismatch discrimination by using molecular becon type probe.

DNA probe to enhance its high binding affinity for the complementary oligoDNAs on CPG plates. (data not shown) Therefore, in this synthesis, the $(CH_2)_{15}$ linker and the $(CH_2O)_6$ spacer were used to increase the stability of DNA duplexes without elimination of DNA probes.

The hybridization affinity of the MB probe **10** for three kinds of target DNAs, that is, wild-type (**11a**: XY = GC), HSC4 mutant (**11b**: XY = AC), and Ca9 mutant (**11c**: XY = GT), was examined by analyzing the strength of fluorescence. It was found by comparison of the wild-type with HSC4 that the MB probe had more than 16-fold fluorescence strength in the C–G match than in the C–A mismatch. The MB probe also exhibited high base discriminating ability (5-fold strength of fluorescence) toward the G–T mismatch. These results showed that the Q, F-labeled MB probe–CPG conjugate in situ synthesized without further purification could be used as a straightforward tool for the SNPs detection on CPG plates.

In summary, we have developed a new hydrophobic linker and a new non-aqueous procedure, $MeNH_2$/THF, for the deprotection of the base and phosphate protecting groups to suppress elimination of oligoDNA probes from CPG. For the first time, we were able to carry out an efficient synthesis of DNA probes without their serious elimination by use of CPG resin. The methods described here would be useful for gene expression analysis and SNPs detection. Further studies are now under way in this direction.

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