Reusable solid-phase supports for oligonucleotides and antisense therapeutics †

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A general method for oligonucleotide synthesis on reusable solid-phase supports has been developed which will significantly lower the cost of large-scale synthesis. It consists of five steps: 1, nucleoside attachment to an hydroxy derivatized support through a *Q-linker* (hydroquinone-*O,O′*-diacetic acid) linker arm; 2, chloro-acetylation of unreacted surface groups; 3, conventional phosphodiester or phosphorothioate oligonucleotide synthesis; 4, cleavage from the support with aqueous or anhydrous ammonia; and 5, support regeneration with methanolic potassium carbonate. The recycling process is fast, fully automatable, and does not require removal of the support from the synthesis column. Fifteen solid-phase supports were evaluated with glycerol-CPG providing the best results. Consecutive syntheses of ISIS 2302, a dGCCCAAGCTGGCATCCGTCA phosphorothioate sequence, on the same synthesis column were performed. A glycerol-CPG synthesis column was satisfactorily used six consecutive times when NH_4OH was the cleavage reagent. However, anhydrous NH_3 allowed twelve consecutive syntheses without any deterioration in support loading, product quality, or amount of product produced. An improved method for preparing the essential nucleoside-3′-hemiesters of the *Q-Linker* and an unexpectedly slower rate of cleavage for phosphorothioate DNA *vs.* phosphodiester DNA are also described.

Introduction

Automated oligonucleotide synthesis on solid-phase supports is a fast and reliable method for preparing small quantities of material.[1] However, demand for synthetic oligonucleotides is growing rapidly, especially for oligonucleotide-based pharmaceuticals.[2,3] These compounds will soon be required in tonne y^{-1} quantities. Successful products must be affordable and so a great deal of effort has been made to reduce the cost of large-scale oligonucleotide synthesis.[4] Originally, large scale oligonucleotide synthesis was prohibitively expensive, but over the last decade costs have decreased dramatically through a combination of increased reagent efficiency, less expensive reagent substitutes, larger synthesizers, and the recovery or regeneration of solvents and reagents.

However, the cost of solid-phase supports has remained relatively unchanged. Previous efforts to reduce the relative cost of these materials have focused on increasing the support's loading capacity.[5–8] Primer HL 30 is one of the supports with high loading (100 μmol g^{-1}) and recently Primer 200 has been introduced with 200 μmol g^{-1} loading. Despite increased loading, Primer-support contributes towards a significant portion of the raw material cost required for synthesis of oligonucleotides. Based on clinical success, we anticipate a surge in the demand for oligonucleotides. As a result, reducing the cost of support is a high priority for us.

Therefore, we have been developing a process to regenerate and reuse solid-phase supports for large-scale solid-phase synthesis. In addition to the cost savings possible by reusing the supports, we sought a recycling process which would be fast, compatible with existing methods and instrumentation, and allow *in situ* processing of the support without having to remove it from the synthesis column or synthesizer. High throughput, small-scale oligonucleotide synthesis may also benefit from rapid, *in situ* processing by eliminating the need for manual intervention between synthesis runs.

Support reuse requires restoration of surface functionality and so degradation of the surface by the cleavage conditions (typically aqueous ammonia) is a potential problem, especially with CPG supports. Therefore, it is important to use as mild as possible conditions for oligonucleotide cleavage and surface regeneration. In preliminary work, we replaced succinic acid with the more labile hydroquinone-*O,O′*-diacetic acid (*Q-Linker*) **1** as the linker arm attaching the first nucleoside to the surface of the support. We also used hydroxy supports instead of amino derivatized supports so the attachment would be through more labile ester linkages instead of amides. These milder conditions allowed us to demonstrate the synthesis of up to six different oligonucleotide phosphodiester sequences from a single batch of support.[9] However, this work required removal of the support from the synthesis columns, a harsh support regeneration step using methylamine and ammonium hydroxide, and a relatively slow and inefficient nucleoside reattachment reaction.

In this manuscript, we describe several significant improvements to the recycling process, evaluate 15 different hydroxy derivatized supports, and apply them to the automated synthesis of phosphorothioate antisense oligonucleotides. The improvements include a faster and more cost effective method for synthesizing the nucleoside starting materials with the *Q-linker* on the 3′-position of **2**, a more effective *O*-benzotriazol-1-yl-*N,N,N′N′*-tetramethyluronium hexafluorophosphate (HBTU)–DMAP coupling procedure for adding the first nucleoside to the support without removing the support from the synthesis column or the synthesizer, and a much milder and faster method for regenerating the support's surface using methanolic potassium carbonate. Anhydrous gas-phase

† Electronic supplementary information (ESI) available: CGE data for octathymidine phosphodiester sequences on supports **P1–5**, **P7**, **P9**, and **P11–13** PAGE data for phosphorothioate products, dimethoxytrityl analysis and UV measurements on supports **P1–7**, **P9** and **P15**, step listing for ABI394 DNA synthesizer and CGE and HPLC data for phosporothioate syntheses on support **P3**. See http://www.rsc.org/suppdata/p1/b1/b105089n/

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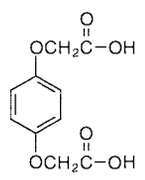
Table 1 Solid-phase supports evaluated. A total of 15 different supports composed of either controlled pore glass (CPG), polystyrene (PS), polymethacrylate (PM) or proprietary resins were evaluated. Both the original (amino or hydroxy) loading of the support and the hydroxy loading after derivatization with the indicated spacer arm are shown. The $t_{90\%}$ value is the time required to release 90% of an ISIS 2302 phosphorothioate sequence from the support using ammonium hydroxide

	Support plus spacer arm	Original Loading μmol g^{-1}	OH Loading μmol g^{-1}	$t_{90\%}$ min
P1	LCAA–CPG, 500 Å + **4**	110	110	9.6
P2	LCAA–CPG, 500 Å + **5**	110	87	6.4
P3	Glycerol-CPG, 500 Å	90	90	5.7
P4	Aminomethyl PS + **5**	30	27	
P5	Aminomethyl PS + **5**	110	80	8.2
P6	Hydroxyethyl PM-PS	100	21	12.7
P7	AF-amino-650 M PM + **5**	180	220	7.0
P8	HW-65F hydroxy PM	1,100	1,100	
P9	HW-65F hydroxy PM + **6**	1,100	110	9.7
P10	Amino TentaGel + **5**	800	110	13.9
P11	Hydroxy TentaGel	270	240	11.0
P12	Amino primer support + **4**	143	320	
P13	Amino primer support + **5**	166	80	
P14	Hydroxy primer support	1000	1000	
P15	Hydroxy primer support + **6**	1000	160	9.7



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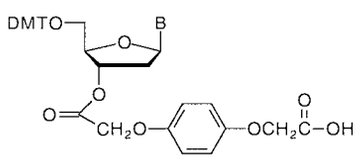

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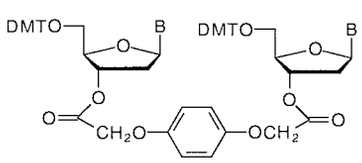

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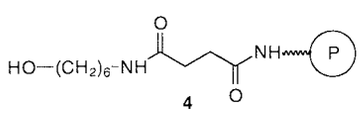

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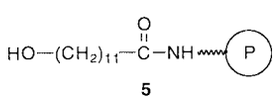

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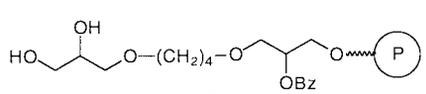

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ammonia cleavage[10] is also shown to dramatically improve the reusability of CPG supports. At least 12 consecutive syntheses of a pharmaceutically important phosphorothioate antisense oligonucleotide (ISIS 2302) were performed without loss of loading capacity or product quality.

We also report an unexpected difference in the rate of cleavage from the support for oligonucleotides with phosphorothioate instead of phosphodiester backbones. This rate is dependent on the type of support and spacer arm employed.

Results and discussion

The hydroquinone-*O,O′*-diacetic acid **1** linker arm has a combination of desirable properties that make it an ideal linker arm for support recycling. It is stable, yet cleaved much faster than a traditional succinic acid linker arm.[11] It is also significantly more reactive than succinic acid in support derivatization reactions.[12] However, our original synthetic route[11] for preparing nucleoside-3′-hemiesters **2**, using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (DEC) and DMAP in pyridine was not ideal for large scale synthesis. Therefore, a less expensive, faster, and more convenient procedure using tosyl chloride and *N*-methylimidazole (NMI) in acetonitrile solution has been developed. This reaction produces similar yields as previous DEC couplings, but avoids pyridine and takes only 10 minutes instead of overnight. Although the symmetrical bifunctional nature of the *Q-linker* leads to some dinucleoside diester **3** as a minor product, this impurity is inert in subsequent esterification reactions. However, if desired, it can be removed by chromatography and used in a transamidation reaction to derivatize amino supports for single use synthesis (data not shown).

Since base hydrolysis of an ester linkage is more facile than an amide linkage, we chose supports with hydroxy surface functionality that resulted in formation of ester linkages. Hydroxy supports can be purchased commercially or else amino supports can be converted into hydroxy supports by adding an additional spacer arm. In this study, Long Chain Alkylamine (LCAA)-CPG, aminomethyl polystyrene, amino polymethacrylate, amino TentaGel and amino Primer Support, were converted to hydroxy functionality by adding either a succinic acid–6-aminohexan-1-ol **4**[9] or a 12-hydroxydodecanoic acid **5**[13] spacer (Table 1). Glycerol CPG **P3**, hydroxyethyl polymethacrylate **P6** and hydroxy Primer Support **P14** were obtained with hydroxy functionality already in place. A butane-1,4-diol diglycidyl ether spacer **6**[14] was used to extend **P6** and **P14** to produce **P9** and **P15**.

Oligonucleotide synthesis cannot begin until the first nucleoside is attached to the solid-phase support. For support reuse, this critical step must be done quickly, efficiently, and reliably in a fully automated fashion. In preliminary work, HBTU and HOBT were used as coupling reagents in a procedure which required the support to be removed from the column and stirred for up to 30 min.[9] Since then, we have examined a variety of uronium and phosphonium coupling reagents for coupling to both amino[15] and hydroxy supports.[12] These studies showed that DMAP was superior to HOBT as a coupling additive, especially for forming ester linkages. Although a number of coupling reagents were satisfactory, HBTU was selected as the reagent of choice because of its low cost and availability in bulk quantities. Using HBTU and DMAP, we were able to reduce the coupling time to only 10 min. Automation was performed by programming an ABI 394 DNA synthesizer with a custom begin procedure to deliver solutions of nucleoside **2** and HBTU–DMAP to the synthesis column.[12] These reagents were stable for at least a week or more on the synthesizer.

We began optimizing the automated nucleoside addition step by performing repetitive cycles of nucleoside attachment, support capping, and nucleoside cleavage without oligonucleo-

Table 2 Multiple octathymidine phosphodiester oligonucleotide syntheses on reusable supports

	Nucleoside loading[a]			Crude product (A_{260} units)			Product purity[b] (% 8-mer)			Average coupling efficiency[b] (%)		
Support	#1	#6	Avg.	#1	#6	Avg.	#1	#6	Avg.	#1	#6	Avg.
P1	84	70	76	79	63	71	82	92	88	97.3	98.8	98.1
P2	69	61	65	49	43	44	85	94	98	97.7	99.1	98.4
P3	94	80	85	50	48	47	69	84	78	94.8	97.7	96.5
P4	25	28	27	29	32	31	96	95	95	99.4	99.3	99.3
P5	57	50	43	26	34	27	90	92	91	98.5	98.8	98.6
P7	61	78	69	44	51	41	94	86	88	99.0	97.8	98.2
P8	220	178	196	112	99	101	71	77	73	95.2	96.3	95.6
P11	153	87	108	90	40	55	88	87	86	98.2	98.0	97.8
P12	156	73	134	69	22	60	74	69	66	95.8	94.8	94.2
P13	46	46	50	22	22	27	94	75	83	99.1	95.9	97.3

[a] Determined by trityl analysis. [b] Determined by CGE (see the supplementary material).

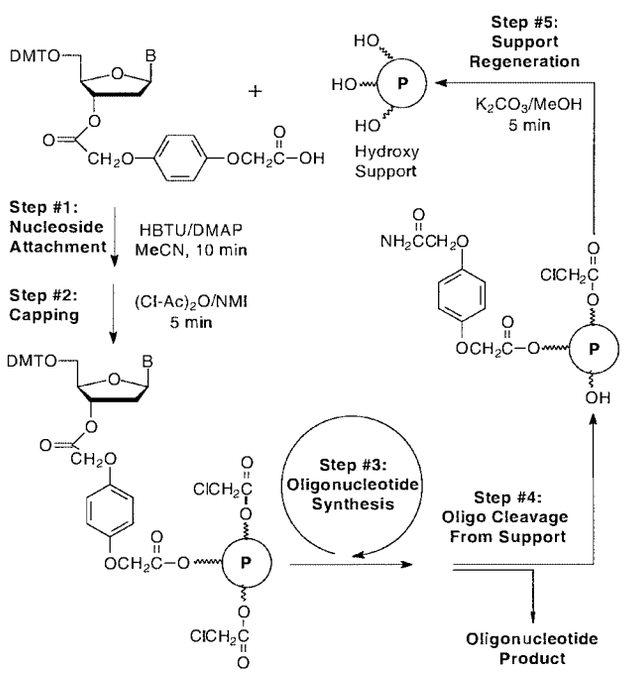
tide synthesis. However, we observed a significant drop (~30%) in nucleoside loading after the first, but not subsequent uses of the support. This decrease remained even when the HBTU–HOBT coupling reagent was replaced with the more potent HBTU–DMAP reagent. We believed the reduced support capacity was due to incomplete removal of the *Q-linker*. However, extended hydrolysis with either NH_4OH or methylamine–NH_4OH solution failed to restore the initial support capacity. Instead, a 5 min treatment with 0.05 M methanolic K_2CO_3 after the NH_4OH cleavage step was required. This non-aqueous reagent has previously been used for deprotection of methylphosphonate modified oligonucleotides.[16] Support surfaces were regenerated by this brief treatment and the amount of nucleoside loaded onto the used supports became nearly identical to the loadings obtained on virgin supports.

Our improved conditions were then applied to oligonucleotide synthesis, using a cycle of nucleoside attachment, support capping, oligonucleotide synthesis, oligonucleotide cleavage, and support regeneration (Scheme 1). Due to the limited number of reagent bottles available on the small scale ABI 394 synthesizer in our lab, the K_2CO_3 support regeneration step had to be performed off the instrument. However, all of the steps are fully automatable and the process shouldn't require removal of the column from large-scale synthesizers with sufficient reagent positions.

A series of six octathymidine $(Tp)_7T$ phosphodiester sequences on a 1 μmol synthesis scale were repetitively prepared using a variety of CPG, polystyrene, polymethacrylate, and polyethylene glycol-polystyrene (TentaGel) supports, *i.e.* each synthesis column was used six consecutive times. As shown in Table 1, the hydroxy group loadings of these supports were quite varied, ranging from ~30 to ~1000 μmol g^{-1}. The $(Tp)_7T$ syntheses were monitored by quantitative dimethoxytrityl analysis, UV quantitation of the crude products, and CGE. In particular, four parameters: nucleoside loading; amount of crude product; overall yield of full-length product; and average coupling efficiency were examined. Results for the first support use (#1), last support use (#6), and the average of all six syntheses (Avg.) are shown for ten supports in Table 2. The CGE analyses confirmed the material produced from each synthesis and provided a quantitative measurement of product quality and consistency (see the supplementary data †).

As each support was reused, a gradual decrease in nucleoside loading was observed for some, but not all, of the supports. This caused a corresponding decrease in the amount of crude product produced. However, as the nucleoside loading decreased, the coupling efficiency increased somewhat and the product purity obtained from the sixth synthesis on the support was sometimes greater than the purity obtained from the first synthesis.

The decrease in loading and yield of product was ~15–20% over six uses for the CPG supports **P1**–**3**. The best results for coupling efficiency, nucleoside loading, and product consistency were obtained with the polystyrene-based supports **P4** and **P5**. The highest nucleoside loadings (178–220 μmol g^{-1}) and product recoveries were obtained from the polymethacrylate support **P8**. The high loading of **P8** resulted in slightly lower coupling yields and product purity than produced by most of the other supports, because our synthesis cycles were not optimized for such high loadings. The TentaGel support **P11** also produced high loadings (87–153 μmol g^{-1}), but was less satisfactory because the results were more variable.



Scheme 1

Supports **P12** and **P13**were unsatisfactory because these were the only supports where the average coupling efficiencies declined as the supports were reused. There was also a large decrease in the amount of product produced as **P12** was reused. Interestingly with **P13**, the amount of product did not vary much, but an unknown "N + 1" impurity increased from 1% to 6.6% as the support was re-used. Although small amounts of this impurity were detected in other octathymidine syntheses, it only increased when **P13** was used. This impurity may have been caused by acrylonitrile alkylation of thymine bases.[17]

During these experiments, we noticed that the capping efficiency after nucleoside derivatization could also be a limiting factor, since some coupling yields (based on trityl analysis) significantly exceeded 100%. These "excess" yields were attributed to phosphoramidite coupling to the surface of the support.[18] Unwanted products build-up on the surface and eventually prevent satisfactory support reuse. The least satisfactory supports

were the Primer Supports **P12–15** that showed up to a 2–3-fold excess of coupling. Excess coupling was also observed to a much smaller extent with the polymethacrylate supports **P7** and **P8**. In these cases, only 10–20% excess coupling was observed in all six syntheses on **P8**, and on two of the syntheses on **P7**. However, despite this observation the crude material obtained from **P8** was still satisfactory and of consistent quality from run to run. The less effective surface capping on supports **P7**, **P8**, and **P12–15** was probably due to their higher surface loadings and short or absent spacer arms. None of the other supports in Table 2 produced any apparent coupling yields >100%.

The results obtained in the above experiments confirmed that a variety of supports could be satisfactorily re-used. However, based on these experiments, we focused our efforts on rigid non-swelling supports that did not have large excess surface functionality (*i.e.* significantly greater than the desired nucleoside loading) and which had a long enough spacer arm to allow effective surface capping. We then wanted to confirm the utility of our recycling strategy for pharmaceutically important phosphorothioate antisense oligonucleotides. Therefore, we began synthesizing the 20-mer phosphorothioate sequence dGCCCAAGCTGGCATCCGTCA, known as ISIS 2302, on reusable hydroxy supports.

However, initial experiments produced much less product than expected. This was because cleavage of the phosphorothioate sequence from the support turned out to be significantly slower than expected. To investigate this, we prepared the ISIS 2302 20-mer sequence as both a phosphodiester and a phosphorothioate oligonucleotide on a conventional single-use LCAA-CPG support. We then studied the rate of cleavage to determine the time required to release 90% of the material from the support ($t_{90\%}$). As expected, cleavage of the phosphodiester sequence was quite fast ($t_{90\%}$ = 1.6 min). Surprisingly, however, under the same conditions the phosphorothioate sequence required almost four times longer for cleavage ($t_{90\%}$ = 5.8 min). We suspected that slower phosphorothioate release was due to a hydrophobic interaction with the support and not slower ester cleavage. This would be similar to elution from solid-phase extraction columns where phosphorothioate sequences require a higher amount of organic modifier for elution than phosphodiester sequences.[19] Evidence for a similar hydrophobic effect during cleavage was obtained when 3 : 1 NH_4OH–ethanol was found to release the phosphorothioate faster than just NH_4OH ($t_{90\%}$ decreased from 5.8 to 4.1 min).

When we examined the cleavage rates for reusable support **P2**, significantly slower rates of phosphorothioate release were also observed, *i.e.* for NH_4OH cleavage the $t_{90\%}$ times for phosphodiester and phosphorothioate modified ISIS 2302 were 2.4 and 9.6 min, respectively. The rate of phosphorothioate release from nine other hydroxy derivatized supports was also determined (Table 1). Most supports had $t_{90\%}$ values between 6–10 min, although the TentaGel support was particularly slow (13.9 min). The variation in $t_{90\%}$ values for different support and linker arm structures was further evidence that oligonucleotide release involves a hydrophobic interaction in addition to hydrolysis.

Therefore, in order to test our recycling process for phosphorothioate production we had to increase the NH_4OH cleavage step from 5 to 15 min. Synthesis columns containing the nine supports, **P1**–**P7**, **P9**, and **P15** were evaluated by performing up to twelve consecutive syntheses of the ISIS 2302 on them. Quantitative dimethoxytrityl analysis was used to determine the nucleoside loading for each synthesis, detect unwanted "excess" coupling (capping failures), and to estimate average phosphoramidite coupling efficiencies (supplementary material). The crude products obtained were quantitated by UV, qualitatively inspected by polyacrylamide gel electrophoresis (PAGE, supplementary material), and analyzed by CGE. †

In each case, full-length ISIS 2302 was obtained but the amount and purity varied with each support. However, in this study, we were more interested in reproducibility of results during support reuse than achieving specific yields or purity. This was because subsequent process optimization could always address product yield and purity. In particular, we monitored the initial nucleoside loading and the amount of crude material produced (Table 3) for consistency over each repetitive synthesis. *Recycling was considered satisfactory if the amount of crude material produced was not less than 90% of the amount produced from the very first use, i.e. from virgin support* (values in bold in Table 3).

The best recycling results were obtained on polystyrene support **P4**. This support gave almost uniform nucleoside loadings (30–38 μmol g^{-1}) through all twelve uses and it wasn't until the last use that the amount of crude product dropped below satisfactory. Supports **P1**–**3**, **P7**, and **P9** also performed well, with reasonably consistent nucleoside loadings for the first seven to ten uses and satisfactory product amounts from four to six uses. Eventually, the performance of each support declined and lower nucleoside loading, lower coupling yields, and reduced product recovery were observed. In some cases, support failure was also evident from an excess coupling yield (>105%) for the first phosphoramidite addition. For example, when **P7** was used the 7th time, the nucleoside loading decreased from 123 to 95 μmol g^{-1}, while the trityl yield of the first coupling reaction increased from 101% to 121%. Support performance then continued to deteriorate as it was further reused. The cause of this is not known, but large excess trityl yields are a general indicator of support recycling failure.

Supports **P6** and **P15** showed inconsistent and unsatisfactory behaviour. **P6** produced continually increasing nucleoside loading and lower product quantity, possibly due to breakdown of the polymethacrylate resin. **P15** produced highly variable nucleoside loadings and only gave satisfactory product quantity one time it was recycled (5th use).

A brown impurity that gradually increased as the supports were reused was also observed. The amount of color varied with the type of support and occurred on both the surface of the supports and, to a lesser extent, in the crude product. This coloration was most pronounced on the surface of **P7** and in the crude product obtained from **P5**. This was attributed to the chloroacetic anhydride capping reagent and could be eliminated by replacing the chloroacetic anhydride reagent with methoxyacetic anhydride. Recycling of supports **P3** and **P9** using methoxyacetic anhydride capping gave good results (Table 3) with 7 and 11 satisfactory support uses, respectively. However, no significant advantage was gained by using the more difficult to prepare methoxyacetic anhydride reagent and we later learned that the brownish color could be avoided by not combining regular acetic anhydride capping (*i.e.* during the oligonucleotide synthesis) with the chloroacetic anhydride capping used after nucleoside addition. Instead, chloroacetic anhydride capping should be used throughout the entire process.

Samples of crude products were analyzed using validated CGE and ion-exchange HPLC methods.[20] This was to verify that the composition of the crude phosphorothioate products produced from multiple support uses was not significantly different from the composition obtained from single support use. The CGE analysis provided single-base resolution and characterization of the amount of full-length product (*N*-mer), failure sequences ($N-1$, $N-2$, *etc.*), and longer ($N+1$, $N+2$, etc.) impurities. An example of the data (from support **P3**) is shown in the supplementary material. In general, the results showed that no products, other than the expected failure sequences, appeared.

Preliminary scale-up experiments using both chloroacetic anhydride and methoxyacetic anhydride were performed using an Amersham Pharmacia Biotech OligoPilot II DNA synthesizer on 200 and 1000 μmol scale. Attempts to use polymethacrylate support **P9** in large packed-bed synthesis columns

Table 3 Crude product obtained from multiple ISIS 2302 phosphorothioate syntheses on reusable supports. Product recoveries within 10% of the amount recovered from virgin support are shown in bold

	A_{260} units produced per gram of support										
Use #	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**	**P9**	**P15**	**P3**[a]	**P9**[a]
1	**7530**	**7630**	**8350**	**3610**	**8010**	**2360**	**12200**	**11500**	**9300**	**8100**	**8480**
2	**7590**	**7680**	**7740**	**4300**	**8330**	3260	**11500**	**11100**	8190	**7750**	**9820**
3	**7530**	**6590**	**8270**	**4220**	**7990**	4520	**12600**	**10600**	7460	**7570**	**10200**
4	**7650**	**6970**	**7970**	**4540**	**7960**	4630	**11700**	**11400**	7620	**7850**	**10900**
5	**6790**	6680	**8120**	**4830**	**7810**	5290	**11600**	**11500**	**8550**	6910	**9490**
6	**6850**	6680	**8120**	**4560**	6720	6420	**11800**	**11400**	7390	**7500**	**10200**
7	5740	6110	7440	**4630**	5690	6610	9880	9980	5900	**7440**	**10300**
8	5930	5170	7370	**4620**	3910		6710			7400	**10700**
9	4750	3460	6690	**4300**	3280		5850			6870	**10000**
10	3640	2420	6170	**3730**	3780		4750			6810	**9190**
11	2780	2700	5190	**3390**	2550		3540			6210	**8450**
12	2350	1850	4660	2750	2050		2550			5220	6170

[a] Methoxyacetic anhydride was used instead of chloroacetic anhydride for capping.

Table 4 Support recycling using gas-phase ammonia cleavage. Preparation of ISIS 2302 on glycerol-CPG **P3** with 500, 1000, or 2000 Å pore sizes.

	Nucleoside loading (μmol g^{-1})			Crude product (A_{260} units g^{-1} of support)		
Use	500 Å	1000 Å	2000 Å	500 Å	1000 Å	2000 Å
1	93.8	47.7	25.5	9490	4270	3230
2	99.7	47.9	26.1	9850	3510	3630
3	96.7	46.8	26.0	9710	4310	3310
4	96.6	47.0	26.4	9640	4270	3630
5	98.9	47.6	26.4	9780	4390	3310
6	100.5	51.7	26.6	9420	4270	4110
7	95.4	50.5	26.3	9710	4140	3550
8	94.9	51.2	26.2	9850	4440	3710
9	94.7	49.7	26.0	10200	4690	3470
10	96.4	54.1	26.1	10200	4690	3390
11	94.7	48.9	26.1	10900	4310	3790
12	97.2	45.6	26.4	10400	4140	3630

failed because of resin swelling. However, glycerol-CPG support **P3** was quite suitable and between five and seven consecutive ISIS 2302 syntheses were performed. These experiments confirmed the scalability of the process, but the results were not optimized to equal the performance of existing manufacturing cycles on single-use Primer HL-30. Instead, we continued to examine other factors that could improve support recycling.

In particular, we knew that prolonged exposure to aqueous NH_4OH had a detrimental effect on the CPG surface and so we examined non-aqueous cleavage conditions. Methanolic K_2CO_3 reagent seemed like a good cleavage reagent because it produced even faster release of phosphorothioates ($t_{90\%} = 1.8$ min) than NH_4OH. Unfortunately, pharmaceutical requirements for low potassium content in antisense drug formulations precluded use of this reagent. Therefore, as an alternative, we tried gas-phase anhydrous ammonia. Gaseous ammonia can be used for both cleavage and base deprotection.[10] However, we wanted a fast turn-around time for the synthesis column and so we only used NH_3 for the cleavage step and base deprotection was performed separately using NH_4OH. Cleavage of the *Q-linker* using ammonia at atmospheric pressure was slow (only 30% in 2 h) and so a Parr pressure reactor at approximately 100 psi and room temperature was required. Under these conditions complete cleavage took place in less than 10 minutes.

Our support recycling procedure was modified to replace the 15 min NH_4OH cleavage step with column drying (vacuum, 5 min), NH_3 cleavage (100 psi, 10 min), and then product elution (methanol–water). Support regeneration with methanolic potassium carbonate and base deprotection with NH_4OH was performed as previously. This procedure was then applied to

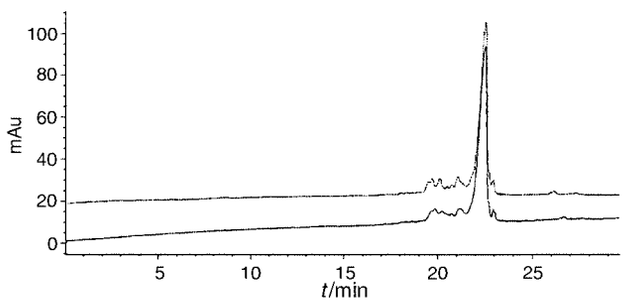


Fig. 1 CGE analyses of ISIS 2302 20-mer phosphorothioate syntheses on 500 Å glycerol-CPG (**P3**) using anhydrous gas-phase ammonia cleavage. Top trace: crude product obtained from the first use of the support (75.3% full-length product). Bottom trace: crude product obtained after twelve consecutive syntheses on the support (73.2% full-length product).

the synthesis of ISIS 2302 on glycerol-CPG with pore sizes of either 500, 1000, or 2000 Å. A dramatic improvement was observed for all three of the supports used (Table 4). Both the nucleoside loading and the quantity of crude product recovered from each consecutive synthesis were very consistent over the course of twelve consecutive syntheses. Indeed, each support continued to function just as well in its twelfth use as it did in its first use.

All twelve Isis 2302 products from the 500 Å glycerol-CPG **P3** synthesis were analyzed by CGE and the average coupling efficiency of each synthesis was calculated from the amount of full-length product. The average coupling efficiencies throughout the twelve syntheses varied by only a small amount, from 98.1% to 98.5%. The CGE traces from the first and twelfth syntheses are superimposed in Fig. 1. The two electropherograms are virtually identical and show average coupling efficiencies of 98.5% and 98.4% for the first and last syntheses, respectively.

Although, these results were extremely encouraging, one complication is the recent discovery that gas-phase deprotection of 2-cyanoethyl phosphate protected oligonucleotides leads to thymine alkylation. The acrylonitrile released by the phosphate protecting groups produces N-3 alkylated thymine impurities.[17] This occurs to a small, but measurable extent, even when only short cleavage times are employed. However, phosphate deprotection prior to cleavage from the support eliminates this side reaction[21] and efforts to totally eliminate this side reaction are in progress.

Conclusions

We have demonstrated the first practical method that permits reuse of solid-supports during oligonucleotide synthesis. This

method is applicable to a variety of different support materials, but works best on rigid supports with surface functional groups in approximately the same capacity as the desired nucleoside loading. In our work, a glycerol-CPG support was preferred because this material was inexpensive, readily available, did not require modification, and was satisfactory in large-scale packed-bed synthesis columns. Although, silica surfaces are sensitive to NH_4OH, we were able to use these supports at least six consecutive times by employing the easily cleaved *Q-linker* linker arm and chloroacetyl or methoxyacetyl capping groups. Anhydrous gas-phase ammonia extends support lifetime to at least 12 uses without any deterioration in performance. Products prepared on the recycled supports were indistinguishable from products produced from single-use supports, although process optimization is still required to match yields from large-scale single-use columns. Even better performance may also be possible with non-silica based supports and evaluation of new synthetic materials is ongoing.

Experimental

Materials and equipment

Hydroquinone-*O,O′*-diacetic acid was purchased from Lancaster Synthesis Ltd. or supplied by Isis Pharmaceuticals. Supports were obtained from CPG Inc. (**P1**–**3**), Applied Biosystems (**P4**), Hamilton (**P5** and **P6**), Supelco (**P7**–**9**), Rapp Polymere (**P10** and **P11**), and Amersham Pharmacia Biotech (**P12**–**15**). HBTU was obtained from Quantum Biotechnologies. DNA synthesis was performed on an ABI 394 DNA synthesizer. Gas-phase ammonia cleavage was done in a Parr pressure reactor. Capillary gel electrophoresis (CGE) for phosphodiester analysis was performed on an HP 3D CE instrument using HP PVA coated capillaries (100 μm × 66.5 cm), HP Oligonucleotide Polymer A, and HP oligonucleotide buffer. Phosphorothioate sequences were analyzed by CGE using J & W Scientific μ-Page 100 polyacrylamide filled capillaries (100 μm × 75 cm) and Tris–urea buffer.

Nucleoside-3′-*O*-hydroquinone-*O,O′*-diacetic acid hemiesters (2)

5′-Dimethoxytrityl-*N*-protected nucleoside **2** (5.0 mmol) and toluene-*p*-sulfonyl chloride (1.86 g, 9.75 mmol) were added to a suspension of hydroquinone-*O,O′*-diacetic acid (1.70 g, 7.5 mmol) in pyridine (3.25 ml) and acetonitrile (50 ml). *N*-Methylimidazole (1.6 ml, 18 mmol) was added with stirring and the mixture dissolved. Monitoring by TLC (5% methanol–$CHCl_3$) showed disappearance of starting nucleoside and formation of slower moving **2** and faster moving diester **3** within 30 min. The solution was evaporated to an oil, redissolved in $CHCl_3$ and washed with 1 M aq. triethylammonium bicarbonate and then water. The $CHCl_3$ was evaporated to a foam containing the crude product. This was purified by silica gel chromatography using 1 : 99 triethylamine–$CHCl_3$ to load the sample, followed by a gradient of 1–5% (1–8% for dG) methanol in 1 : 99 triethylamine–$CHCl_3$. Yield: 54–67%.

Automated nucleoside attachment

Acetonitrile solutions of 0.15 M diisopropylethylamine–nucleoside **2** and 0.15 M HBTU–DMAP were installed on spare base positions. 1 M Chloroacetic anhydride–THF and 2 M *N*-methylimidazole–1 M 2,6-lutidine–THF were installed as Cap A and B reagents. Accurately weighed hydroxy support (10–15 mg) sealed into a synthesis column was installed. An automated "Begin" procedure containing a custom function to simultaneously deliver both nucleoside and HBTU solutions to the synthesis column was used. This procedure performed a 10 min coupling step followed by a 5 min capping step to block underivatized hydroxy sites (see supplementary material†).

Octathymidine phosphodiester synthesis

A standard 1 μmol scale synthesis cycle was modified to compensate for the high surface loadings of the supports used by increasing the phosphoramidite concentration from 0.1 M to 0.2 M and the coupling wait time from 60 s to 120 s. Dimethoxytrityl cations were collected, diluted to 50.0 ml, and quantitated at 504 nm to determine nucleoside loadings. CGE analysis was performed to determine overall and average coupling efficiencies.

Phosphorothioate synthesis

As above, but 0.1 M 3-oxo-3*H*-1,2-benzodithiolyl 1,1-dioxide was used as sulfurization reagent in a phosphorothioate synthesis cycle.

Cleavage from the support

After oligonucleotide synthesis (trityl-off), products were automatically cleaved from the supports by either a 5 min (phosphodiester sequences) or 15 min (phosphorothioate sequences) NH_4OH treatment. Alternatively, the synthesis column was briefly dried (5 min) under vacuum, treated with NH_3 (100 psi, 10 min) in a Parr reactor, and the product eluted in methanol (1 ml) and then water (2 ml). Base deprotection was performed in NH_4OH (55 °C, 16 h).

Support regeneration

After oligonucleotide cleavage, the synthesis columns were manually filled with 0.05 M K_2CO_3–methanol using a syringe and left with occasional agitation for 5 min. The columns were rinsed with methanol (10 ml), dried under vacuum (5 min), reinstalled on the synthesizer and washed with anhydrous acetonitrile (2 min).

References

1 M. H. Caruthers, *Science*, 1985, **230**, 281.
2 S. T. Crooke, *Biochim. Biophys. Acta*, 1999, **1489**, 31.
3 S. Agrawal and E. R. Kandimalla, *Mol. Med. Today*, 2000, **6**, 72.
4 Y. S. Sanghvi, M. Andrade, R. R. Deshmukh, L. Holmberg, A. N. Scozzari and D. L. Cole, in *Manual of Antisense Methodology*, eds. G. Hartmann and S. Endres, Kluwer Academic Publishers, Boston, 1999, pp. 3–23.
5 P. Wright, D. Lloyd, W. Rapp and A. Andrus, *Tetrahedron Lett.*, 1993, **34**, 3373.
6 P. Jaisankar, M. Hinz, E. Happ and H. Seliger, *Nucleosides, Nucleotides*, 1998, **17**, 1787.
7 K. L. Fearon, B. L. Hirschbein, C.-Y. Chiu, M. R. Quijano and G. Zon, *Oligonucleotides as Therapeutic Agents, Ciba Found. Symp.*, 1997, **209**, 19.
8 A. K. Patnaik, N. S. Rao, P. Kumar, A. K. Sharma, B. S. Garg and K. C. Gupta, *Helv. Chim. Acta*, 2000, **83**, 322.
9 R. T. Pon, S. Yu, Z. Guo and Y. S. Sanghvi, *Nucl. Acids Res.*, 1999, **27**, 1531.
10 J. H. Boal, A. Wilk, N. Harindranath, E. E. Max, T. Kempe and S. L. Beaucage, *Nucleic Acids Res.*, 1996, **24**, 3115.
11 R. T. Pon and S. Yu, *Nucleic Acids Res.*, 1997, **25**, 3629.
12 R. T. Pon, S. Yu and Y. S. Sanghvi, *Bioconjugate Chem.*, 1999, **10**, 1051.
13 A. Nanthakumar, R. T. Pon, A. Mazumder, S. Yu and A. Watson, *Bioconjugate Chem.*, 2000, **11**, 282.
14 G. T. Hermanson, *Bioconjugate Techniques*, Academic Press, San Diego, 1996.
15 R. T. Pon and S. Yu, *Synlett*, 1999, 1778.
16 I. Habus, T. Devlin, R. P. Iyer and S. Agrawal, *Bioorg. Med. Chem. Lett.*, 1996, **6**, 1393.
17 M. Mag and J. W. Engels, *Nucleic Acids Res.*, 1988, **16**, 3525.
18 R. T. Pon, N. Usman and K. K. Ogilvie, *Biotechniques*, 1988, **6**, 768.
19 M. Gilar and E. S. P. Bouvier, *J. Chromatogr. A*, 2000, **890**, 167.
20 G. S. Srivatsa, P. Klopchin, M. Batt, M. Feldman, R. H. Carlson and D. L. Cole, *J. Pharm. Biomed. Anal.*, 1997, **16**, 619.
21 C. A. Chang and T. Horn, *Nucleosides, Nucleotides*, 1999, **18**, 1205.