

Tocopherol (Vitamin E) Modified Oligonucleotides II: Utilising Hydrophobicity to Aid Purification

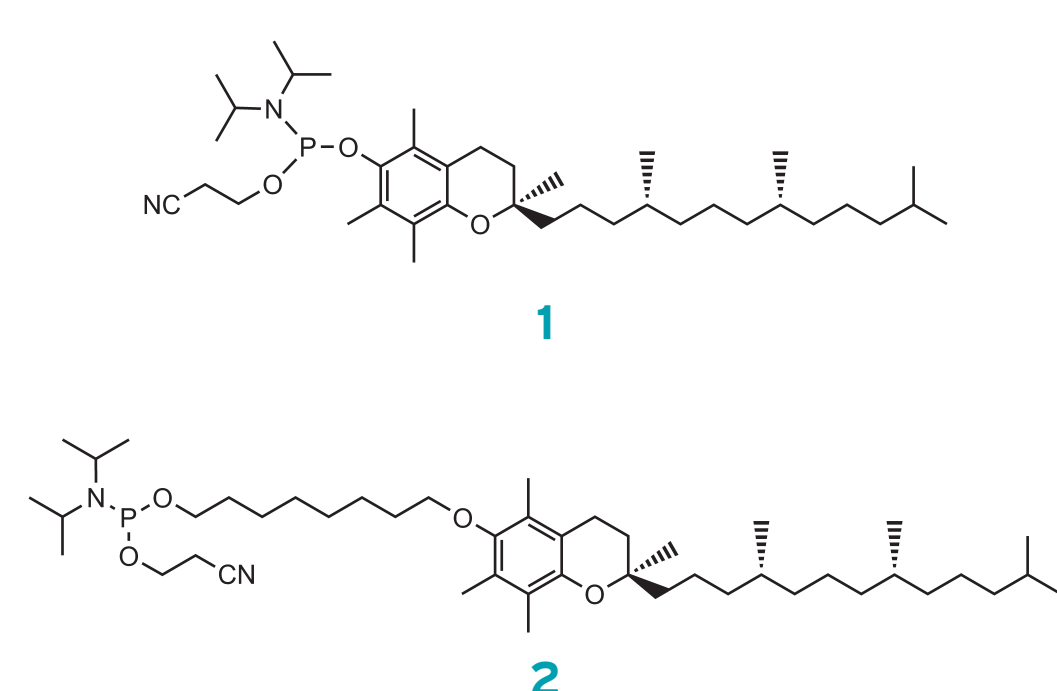
Sheena Aitken, Ricky Archer, Grant McGeoch, Catherine McKeen and Douglas Picken; Link Technologies Ltd, Bellshill, UK.

catherine@linktech.co.uk

Introduction

As with cholesterol modifications, tocopherol has been shown to have potential use in the delivery of oligonucleotides into cells. Vitamins such as tocopherol are not produced by the target cells, but are used by the latter and therefore vitamins are recognised. They are thought to be internalised by cells only after interaction with a binding protein and therefore have the potential for specific targeting of a cell type.^{1,2,3,4}

The hydrophobic nature of tocopherol has also been utilised as a means of improving the purification of ribozymes.⁵ As an extension to previous work⁶, therefore, we have investigated the use of tocopherol CEP (1) and octyltocopherol CEP (2) as a means of allowing an initial purification of thiol-modified oligos with a view to improving the efficiency of a second ion-exchange purification.



Thiol-modified oligonucleotides are often used as a means of incorporating labels such as dyes by reaction with maleimides or acetamides. There is often a need to carry out multiple purifications in order to ensure the purity of the thiol-modified oligonucleotide prior to conjugation and a simple, efficient method of removing DMT-containing N-1 failures would be advantageous.

Our aims were three-fold, to show: (1) tocopherol is sufficiently hydrophobic that a tocopherol-modified oligo will be retained on a TOPS-DNA cartridge; (2) purification by TOPS-DNA cartridge of tocopherol-S-S-oligonucleotides result in a purer thiol-modified oligo than a standard TOPS-DNA purification of a DMT-S-S-oligo; and (3) there is no adverse effect on the functional use of the thiol-modified oligo by using tocopherol to enhance purification.

Experimental

Synthesis Conditions

Synthesis was carried out on an ABI 394 DNA/RNA automated synthesiser using the following reagent specifications: standard CEPs (Bz-dA, dmf-dG, Ac-dC and dT) - 0.1M in acetonitrile, coupling time = 30s; tocopherol CEPs (1 and 2) - 0.1M in anhydrous, alcohol-free, dichloromethane, coupling time = 900s; solid support - dT 1000Å SynBase™ 1µmol column; activator - 0.25M ETT; cap A - THF/pyridine/acetic anhydride (8:1:1); cap B - 10% methylimidazole in THF; oxidiser - 0.02M iodine in THF/pyridine/water (7:2:1); deblock - 3% TCA/DCM.

Deprotection Conditions

All oligonucleotides were cleaved and deprotected using AMA at room temperature for 2h.

Analysis Conditions

IE-HPLC was carried out on a Dionex DNA-pac PA100, 4.5x250mm column using Buffer A: 50mM Tris, 0.1M ammonium chloride, 20% acetonitrile, pH 7.4 and Buffer B: 50mM Tris, 1.0M ammonium chloride, 20% acetonitrile, pH 7.4 over a gradient of 5-20% B over 2min then 20%-100% over 20min at a flow rate of 1ml/min.

LCMS was carried out on an Agilent 6220 Accurate Mass TOF LCMS with 1260 pumps and DAD, using a Zorbax C18, 3.5µm, 2.1x300mm column, Buffer A: 190mM HFIP, 7mM TEAA, 5% MeOH in water, Buffer B: MeOH, with a gradient of 0-100% B over 12min. All samples were at a concentration of 20D/ml.

1 Delivery of oligonucleotides and analogues: The oligonucleotide conjugate-based approach, F. Marlin, P. Simon, T. Saison-Behmoaras and C. Giovannangeli, *ChemBioChem*, 11, 1493-1500, 2010.

2 Efficient in vivo delivery of siRNA to the liver by conjugation to alpha-tocopherol, K. Nishina, T. Unno, Y. Uno, T. Kubodera, T. Kanouchi, H. Mizusawa and T. Yokota, *Mol. Ther.*, 16, 734-740, 2008.

3 Resolution of liver cirrhosis using vitamin-A coupled liposomes to deliver siRNA against a collagen-specific chaperone, Y. Sato, K. Murase, J. Kato, M. Kobune, T. Sato, Y. Kawano, R. Takimoto, K. Takada, K. Miyayoshi, T. Matsunaga, T. Takayama and Y. Niitsu, *Nat. Biotechnol.*, 26, 431-442, 2008.

4 Attachment of vitamin E derivatives to oligonucleotides during solid-phase synthesis, D. Will and T. Brown, *Tet. Letts.*, 33, 2729-2732, 1992.

5 Fast and simple purification of chemically modified hammerhead ribozymes using a lipophilic capture tag, B.S. Sproat, T. Rupp, N. Menhardt, D. Keane, and B. Beljer, *Nucleic Acids Res.*, 27, 1950-1955, 1999.

6 Tocopherol (vitamin E) modified oligonucleotides, S. Aitken, R. Archer, G. McGeoch, C. McKeen and D. Picken, poster presented at TIDES 2011. Available to download from http://www.linktech.co.uk/support/knowledge_base/419_tocopherol-vitamin-e-modified-oligonucleotides-tides-2011-poster.

7 While this was the only activator used for this work, 0.5M ETT, 0.3M BTT and 0.25M DCI have all been used to successfully couple both tocopherol derivatives 1 & 2 to oligonucleotides.

Results & Discussion

In all cases the oligonucleotide sequence was TTC GGC TTG TCC GTG GAA TCT CAC AGC TTA T where modifications were incorporated at the 5' end.

Is tocopherol sufficiently hydrophobic to be retained on a TOPS column?

5'-Tocopherol (1) modified oligonucleotides were synthesised and analysed by LCMS (RT = 9.15min, Calc. MW = 9954.89, Obs. MW = 9965 - 9967). These were loaded onto TOPS cartridges and the standard protocol, modified to contain a TCEP treatment step, was carried out on both. Comparing the yield prior to purification (~400 OD/ml) to that after purification (0.5 - 2.5 OD/ml) indicates that tocopherol is sufficiently hydrophobic to hold the oligonucleotide onto the cartridge.

Can this hydrophobicity be utilised to improve the purification of thiol modifications by cartridge?

The same 31mer mixed DNA sequence was used as before where the thiol modifier (3) was used to incorporate O-C6-S-S-C6-O between the oligonucleotide and tocopherol (1) as shown in Figure 1. As a control the same oligonucleotide sequence was modified with the thiol modifier alone.

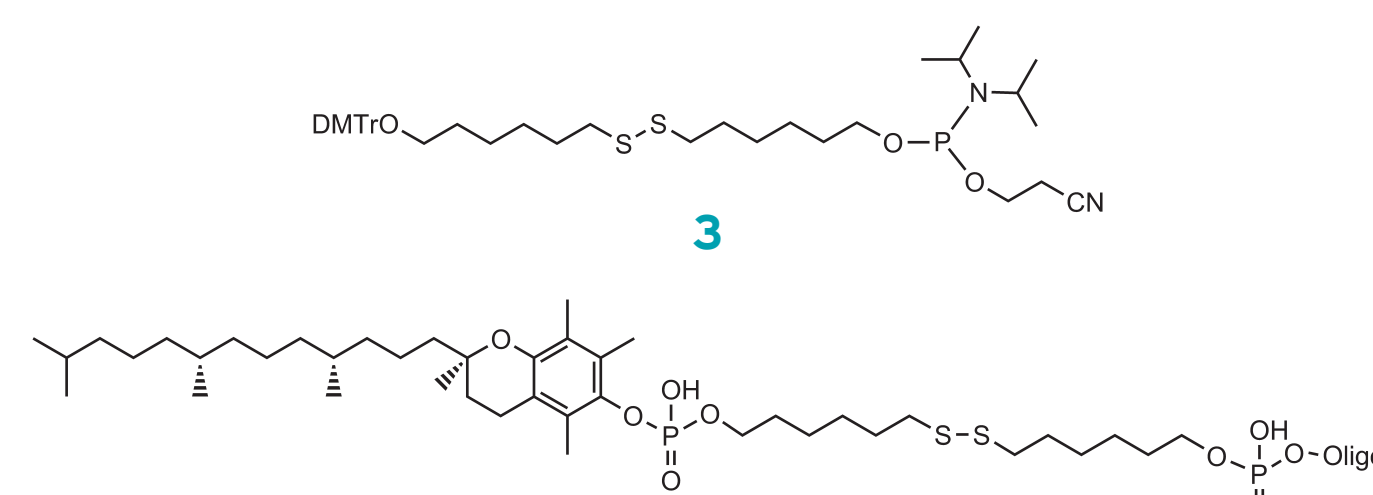


Figure 1. Tocopherol-thiol-modified oligonucleotide.

After synthesis the oligonucleotides were cleaved and deprotected in AMA for 2h at room temperature then immediately loaded onto a Varian DNA TOPS column, with the exception of oligonucleotide 6 which was treated with 100mM TCEP and passed through a G25 sephadex column. The methods used are summarised in Table 1.

Table 1. TOPS purification methods.

Method ID	Oligo before TOPS	Modification to standard TOPS protocol
1	Toc-O-C6-SS-C6-oligo	100mM TCEP treatment after TFA treatment then oligo elution.
2	Toc-O-C6-SS-C6-oligo	Elution after TFA treatment, 100mM TCEP treatment, elution of thiol-modified oligo.
3	Toc-O-C6-SS-C6-oligo mixed with DMT-O-C6-SS-C6-oligo	Elution after TFA treatment, 100mM TCEP treatment, elution of thiol-modified oligo.
4	DMT-O-C6-SS-C6-oligo	No modification
5	DMT-O-C6-SS-C6-oligo	TFA treatment replaced with 100mM TCEP treatment
6	HO-C6-SS-C6-oligo	No cartridge purification, 100mM TCEP treatment only followed by G25 sephadex.

In all cases the desired thiol-functionalised oligonucleotide was obtained. RP-HPLC in general showed two peaks, one being HS-C6-O-oligonucleotide and the other being attributed to oligonucleotide-O-C6-S-S-C6-O-oligonucleotide which was confirmed by LCMS.

Examples of the IE-HPLC analysis of the thiol-functionalised oligonucleotides are shown in Figures 2-5, including expansions around the main peak to show the N- failures. Although the standard method gives a relatively clean product (Figure 2) when compared with the unpurified thiol-modified oligonucleotide (Figure 5), methods 2 and 3 showed less N- failures. It is interesting to note that while method 5 gave similar quality products to the standard TOPS purification method, the dimerised product oligonucleotide-O-C6-S-S-C6-O-oligonucleotide was always observed by HPLC. This was also the case where the crude oligonucleotide was only treated with TCEP and passed through a G25 column. It is also interesting that the oligonucleotides purified utilising tocopherol's hydrophobic properties show peaks with a slightly longer retention time than the main peak. Possibly there are some tocopherol containing oligonucleotides being eluted from the purification cartridge, but in any case this does not interfere with further purification of the oligonucleotide.

Does the use of tocopherol have any adverse reaction on the labelling efficiency of the thiol-functionalised oligonucleotide?

Labelling of the HS-C6-O-oligonucleotides with 6-iodoacetamidofluorescein (6-IAF) was carried out and, in all cases, the desired product was obtained. The labelled product has a later retention time than the N+ (possibly tocopherol containing) failures.

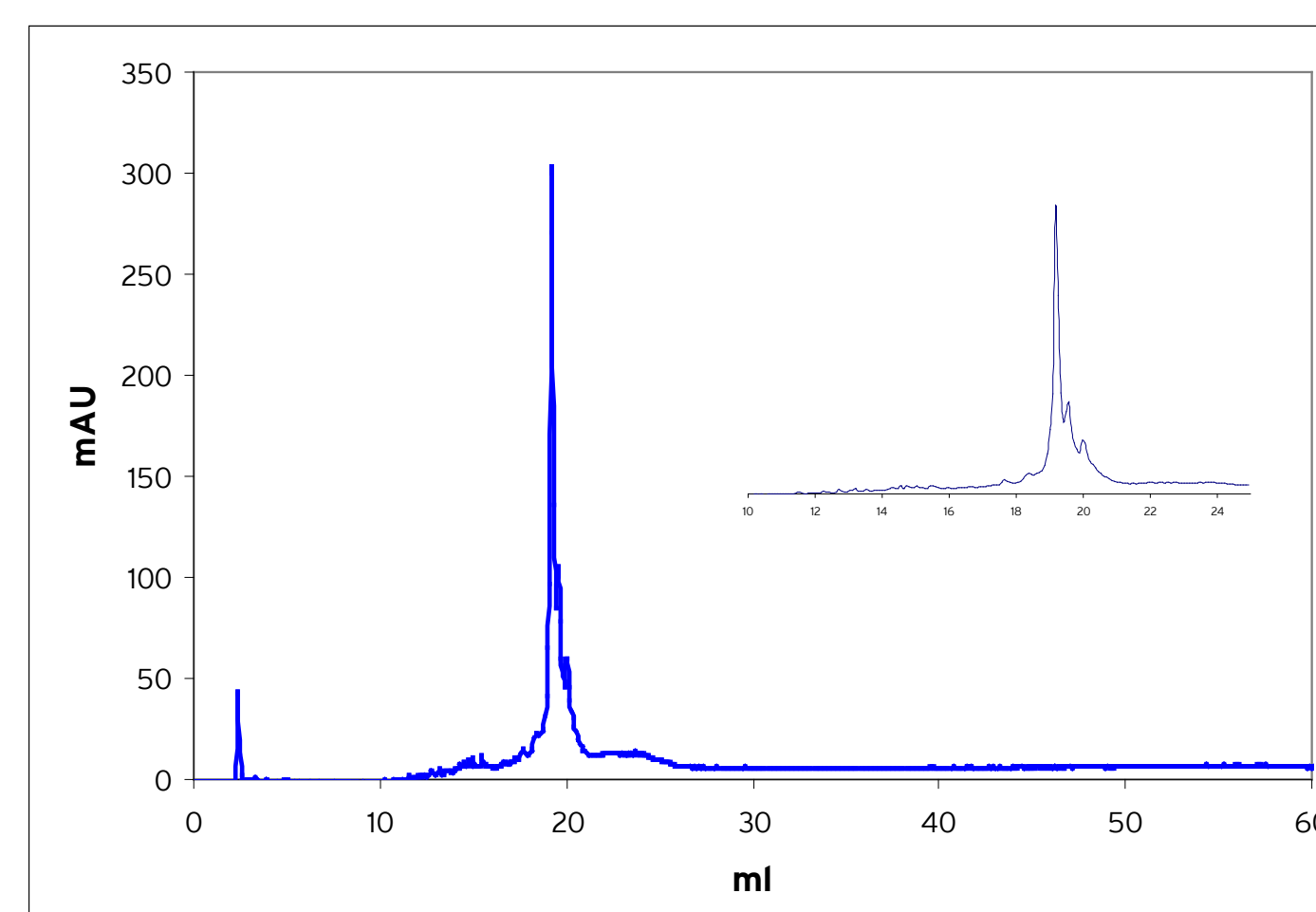


Figure 2. IE-HPLC data of thiol-modified oligonucleotide purified using method 4.

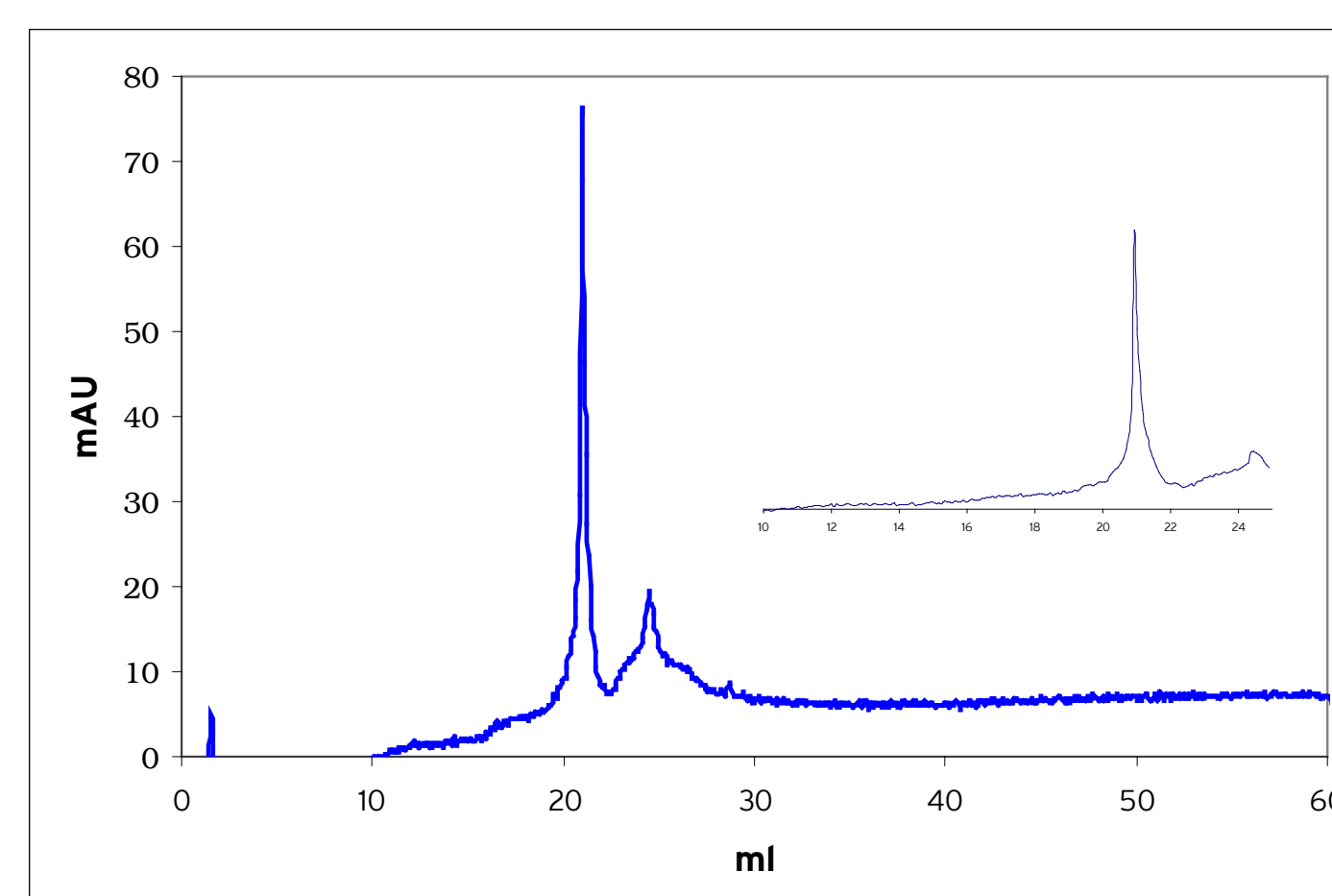


Figure 3. IE-HPLC data of thiol-modified oligonucleotide purified using method 3.

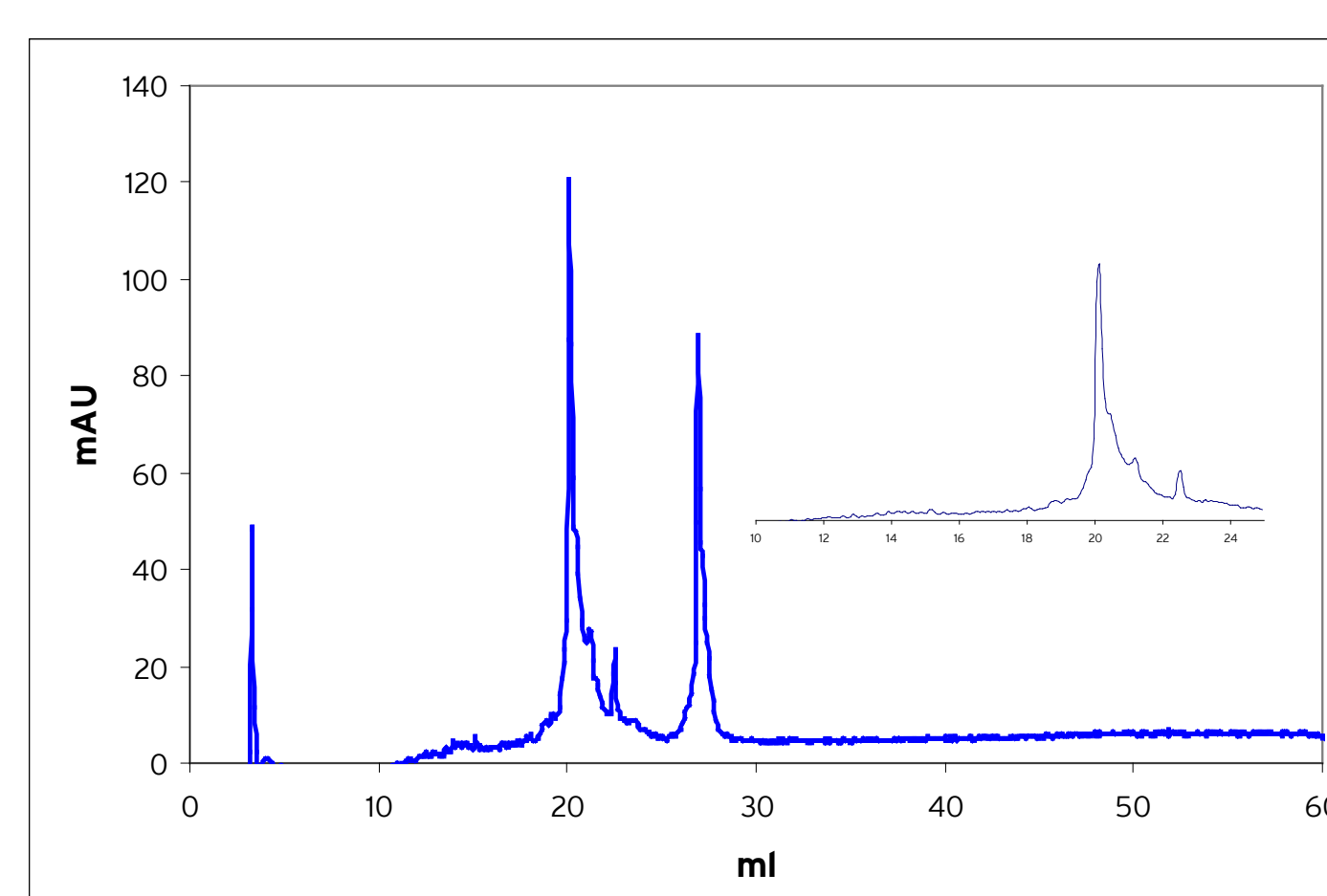


Figure 4. IE-HPLC data of thiol-modified oligonucleotide purified using method 5.

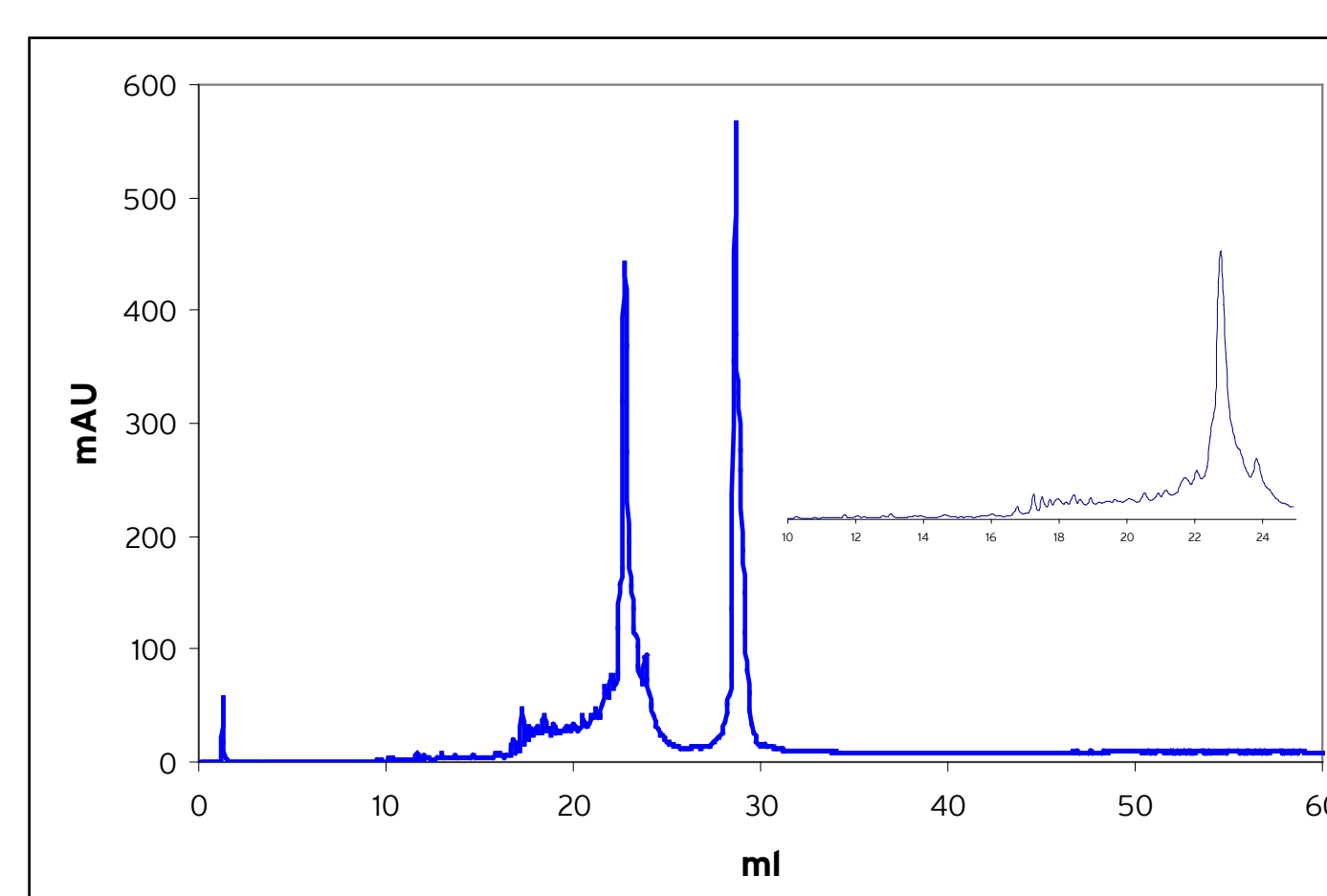


Figure 5. IE-HPLC data of thiol-modified oligonucleotide purified using method 6.

Conclusions

Tocopherol is sufficiently hydrophobic that a tocopherol-modified oligo will be retained on a TOPS DNA cartridge.

Purification by TOPS DNA cartridge of tocopherol-S-S-oligonucleotides results in a purer thiol-modified oligo than a standard TOPS DNA purification of a DMT-S-S-oligonucleotide.

Switching detritylation for disulphide reduction using DMT-S-S-oligonucleotide is an alternative method but does not improve the purity of the thiol-functionalised oligonucleotide in comparison to the standard TOPS method.

Thiol-modified oligonucleotides that have been purified utilising the hydrophobicity of tocopherol label efficiently with 6-iodoacetamidofluorescein (6-IAF).

Further Information

Any queries regarding this work should be directed to Dr Catherine McKeen, Product Manager, Link Technologies Ltd.

www.linktech.co.uk

