

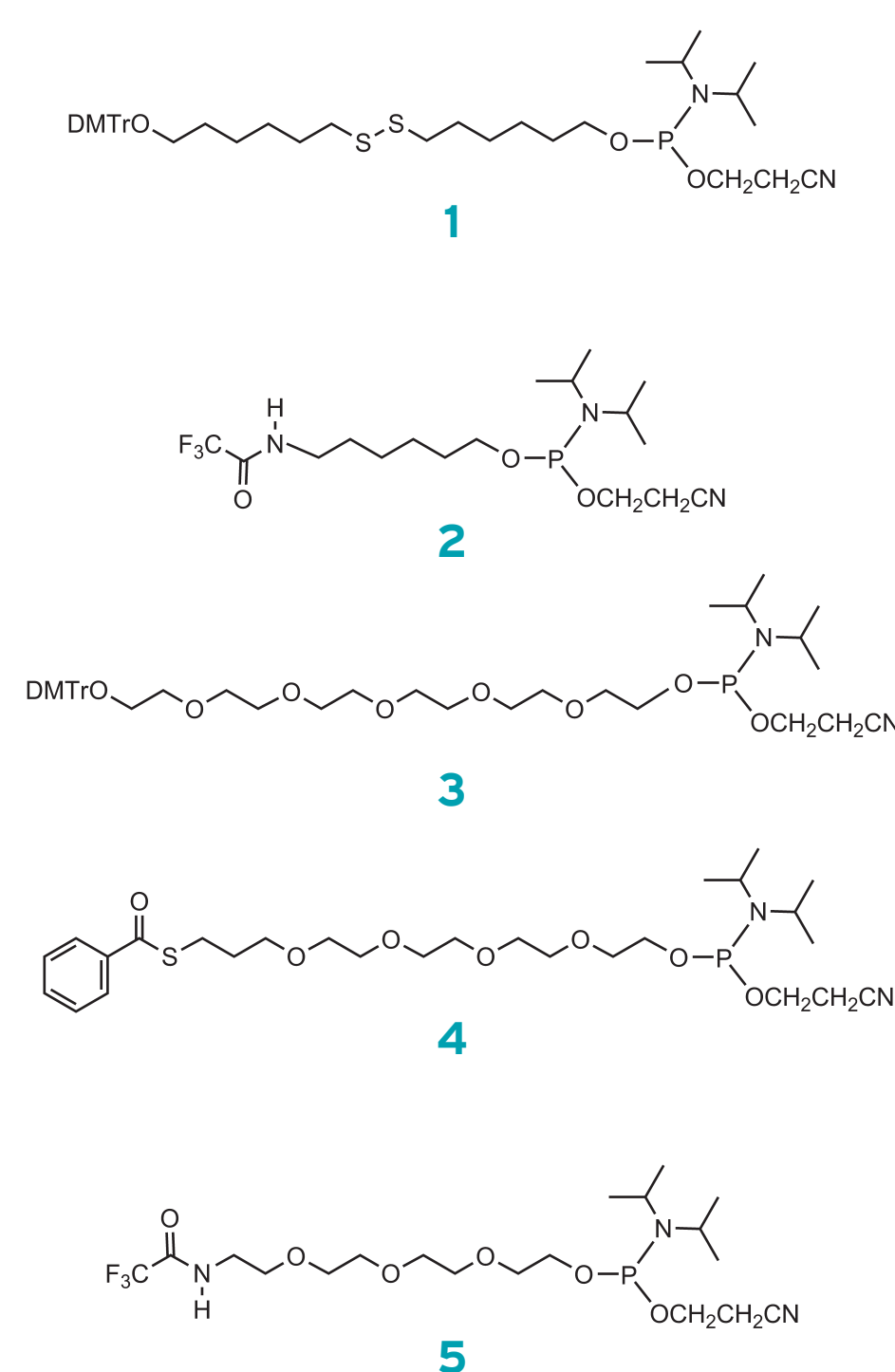
# A Comparison of Hydrophilic and Hydrophobic Amino and Thiol Linkers for use in Post-Synthetic Labelling of Oligonucleotides

Sheena Aitken, Ulrich Ixkes, **Catherine McKeen** and Douglas Picken; Link Technologies Ltd, Bellshill, UK.

[catherine@linktech.co.uk](mailto:catherine@linktech.co.uk)

## Introduction

Thiol<sup>1,2</sup> and amino<sup>3,4</sup> linkers such as **1** and **2** are widely used as a means of post-synthetically labelling oligonucleotides. In general these are hydrophobic in nature but are often used in conjunction with hydrophilic spacers such as HEG (spacer 18) (**3**) and spacer 9. There now exist hydrophilic thiol (**4**) and amino (**5**) linkers that alleviate the need for the additional hydrophilic spacer. The use of these in post synthetic labelling of oligonucleotides with HRP, fluorescein and thioctic acid has been evaluated.



## Experimental

### Synthesis Conditions

All oligonucleotides were synthesised on an ABI 394 DNA/RNA synthesiser with 15min coupling for the addition of the modifications and 30s for standard amidite coupling. The amidites were used at 0.1M

**1** Chemical synthesis of oligonucleotides containing a free sulphhydryl group and subsequent attachment of thiol specific probes, B.A. Connolly and P. Rider, *Nucleic Acids Research*, **13**, 4485-4502, 1985.

**2** The preparation and application of functionalised synthetic oligonucleotides: III. Use of H-phosphonate derivatives of protected amino-hexanol and mercapto-propanol or -hexanol, N.D. Sinha and R.M. Cook, *Nucleic Acids Research*, **16**, 2659-2670, 1988.

**3** The synthesis of oligonucleotides containing a primary amino group at the 5'-terminus, B.A. Connolly, *Nucleic Acids Research*, **15**, 3131-3139, 1987.

**4** R.I. Hogrefe, H. Mackie, M.L. Powell, *Amer. Biotechnologies News Ed.*, **6**, 47, 1988.

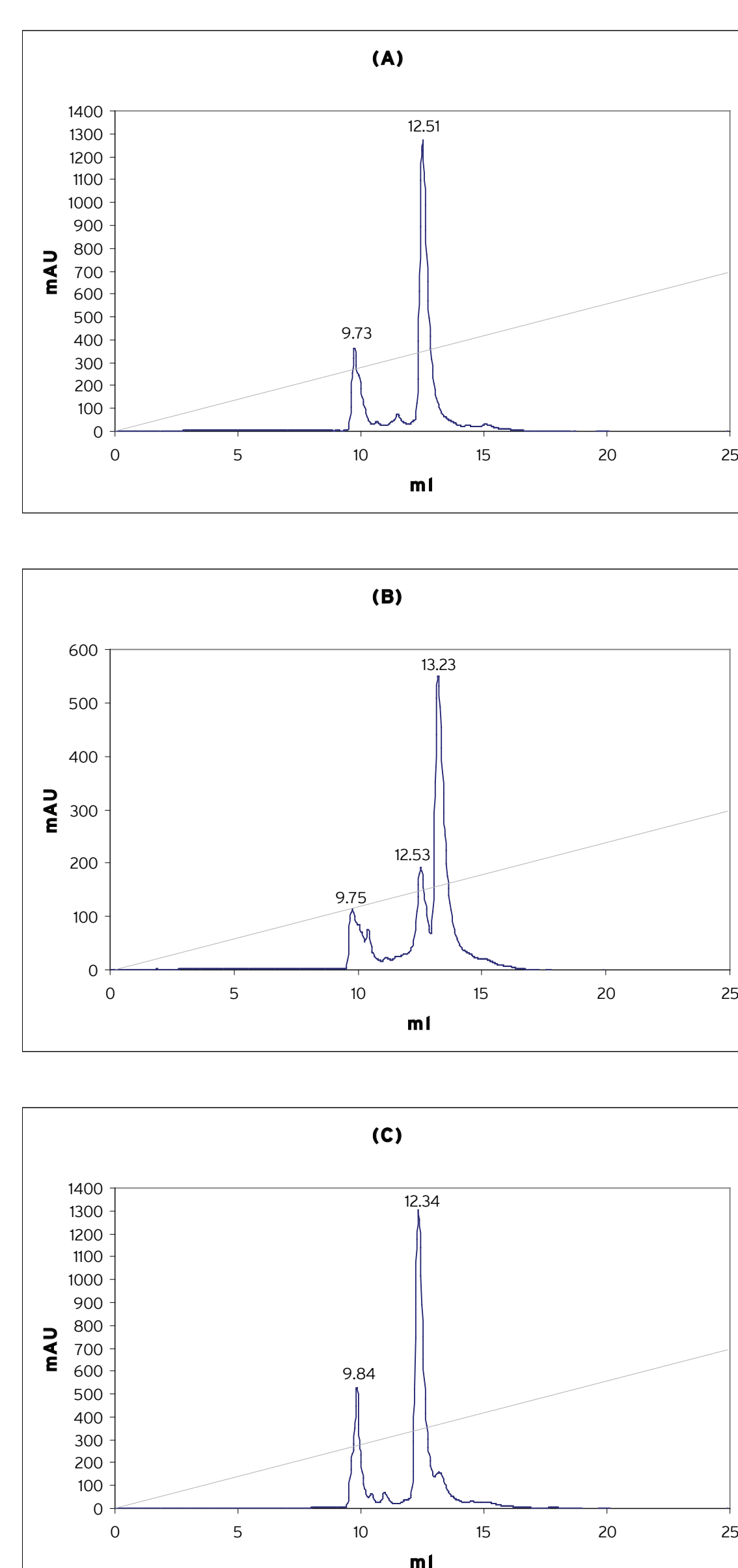


Figure 2. HPLC data of thioctic acid labelled oligos: (A) amino-C6; (B) amino-C6-HEG; (C) amino-11.

concentrations in acetonitrile, with 0.1M BTT or 0.25M DCI in MeCN as the activator. NOTE: thiotetrazoles were not used with the BzS-TEG modification since this gives a side reaction. The mechanism of this is currently being investigated. All supports used were 1000Å CPG.

### Deprotection Conditions

All oligonucleotides were treated with 20% diethylamine/ acetonitrile prior to cleavage and deprotection, which was carried out with ammonia at room temperature overnight.

### Conjugation Conditions

All thiol-modified oligonucleotides were treated with 87mM TCEP in water immediately prior to conjugation. The excess reducing reagent was removed by G25 and the conjugation buffer used was 0.1M sodium phosphate, pH 7.4.

All amino-modified oligonucleotides were conjugated in 0.1M sodium carbonate/bicarbonate buffer, pH 9.75.

### Analysis Conditions

RP-HPLC: Agilent Eclipse XBD-C18 5µm, 4.6x150mm column using buffer A: 0.1M TEAA and buffer B: 100% MeCN with a gradient of 0-50% B for 25min (1ml/min).

MALDI-TOF: Bruker Ultraflex or Autoflex using a 3-hydroxypicolinic acid matrix. This data was provided by EGT-SA.

IE-HPLC: ABI Poros HQ/20 4.6mmDx100mmL column, buffer A = 20mM sodium phosphate buffer, pH 6.0, buffer B = 20mM sodium phosphate/1M sodium chloride buffer, pH 6.0.

All oligonucleotides were analysed and used unpurified.

## Results & Discussion

The same three sequences were used throughout:

17mer; TCT AAG TGG CGA CCA TT (used for HRP conjugations);

10mer; TTT TTT TTT T (used with 6-IAF couplings);

31mer; TTC GGC TTG TCC GTG GAA TCT CAC AGC TTA T (used for all other post labelling reactions).

### Thiol Modifier Comparison

Oligonucleotides modified with BzS-TEG (**4**), thiol S-S C6 (**1**), and a combination of thiol S-S C6 (**1**) and HEG (**3**) were synthesised. One set was coupled to fluorescein using fluorescein maleimide and another to horseradish peroxidase (HRP) enzyme modified with N-[β-Maleimidopropoxy]succinimide ester (BMPS). Fluorescein labelling using 6-iodoacetamido fluorescein (6-IAF)

was also carried out. The results are summarised in Table 1 below.

For the purpose of the comparisons, the HRP couplings were carried out with a 1:1 ratio of HRP to thiol-modified oligonucleotide. Normally a higher number of equivalents of HRP would be used. In all cases the conjugated product was observed. The MS data is shown below in Figure 1, the worst result being the oligonucleotide modified with thiol C6 S-S (**1**) where the product is barely visible. For the BzS-TEG (**4**) and thiol C6 S-S (**1**)/HEG (**3**) modified oligonucleotides, the conjugation was more obvious and were comparable to each other with the latter giving slightly better coupling which may be due to the longer distance between the thiol functionality and the oligonucleotide. The enzyme conjugation work is being repeated but this time with a higher HRP:thiol oligonucleotide ratio and using SMCC rather than BMPS.

Conjugations to maleimides and iodoacetamides showed a similar pattern in that thiol C6 S-S (**1**) showed lower coupling than BzS-TEG (**4**) and thiol C6 S-S (**1**)/HEG (**3**), with these latter being comparable.

### Amino Modifier Comparison

The amino-modified oligonucleotides were all labelled with thioctic acid via the NHS ester. The results are summarised in Table 2 below. There was no real difference in the post-labelling efficiency between the three amino-modified oligonucleotides although the amino-C6 (**2**)/HEG (**3**) combination gave slightly higher labelling which may be due to the longer distance between the amino functionality and the oligonucleotide. The HPLC data for each of the thioctic acid labelling reactions is shown in Figure 2.

## Conclusions

The use of hydrophilic amino-11 modifier (**5**) and BzS-TEG (**4**) phosphoramidites proved an efficient means of incorporating a hydrophilic linker into an oligonucleotide without the need for an additional hydrophilic spacer such as HEG (**3**). The post-labelling efficiency is comparable with the current most commonly used amino (**2**) and thiol linkers (**1**) used on their own or in combination with HEG (**3**). BzS-TEG is not compatible with thiotetrazoles as activators therefore DCI is recommended for this modification.

## Further Information

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

[www.linktech.co.uk](http://www.linktech.co.uk)

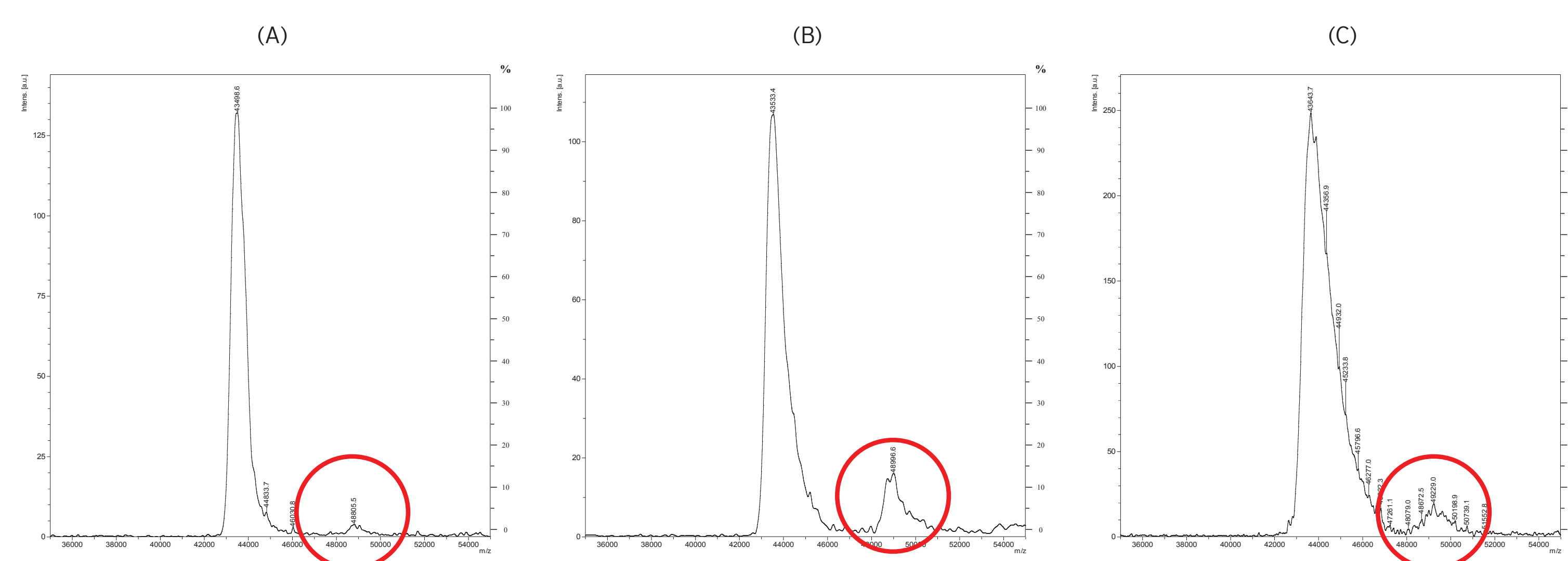


Figure 1. MS data of HRP-oligo conjugation reactions: (A) thiol-C6 S-S; (B) thiol-C6 S-S-HEG; (C) Bz-thiol-TEG. The conjugation product is circled.

Table 1. Summary of coupling reactions to thiol modified oligos.

Linker	Spacer	Conjugated to:	% Labelled	Ratio of HRP-oligo : unreacted HRP	MW calc.	MW obs.
<b>1</b>		Fluorescein Maleimide	70.31		10072	10097
<b>1</b>	<b>3</b>	Fluorescein Maleimide	86.76		10416	10438
<b>4</b>		Fluorescein Maleimide	82.86		10207	10254
<b>4</b>		6-IAF	83.87		3669	3703
<b>1</b>		HRP		1.44 : 98.56	48872	48806
<b>1</b>	<b>3</b>	HRP		8.22 : 91.78	49215	48997
<b>4</b>		HRP		5.02 : 94.98	49006	49229

Table 2. Summary of coupling reactions to amino modified oligos.

Linker	Spacer	Conjugated to:	% Labelled	MW calc.	MW obs.
<b>2</b>		Thioctic acid	78.54	9816	9846
<b>2</b>	<b>3</b>	Thioctic acid	80.79	10160	10178
<b>5</b>		Thioctic acid	76.71	9892	9919

