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^{18}F-Labelling of Oligonucleotides Using Succinimido 4-[^{18}F]Fluorobenzoate

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A general method for the labelling of oligodeoxynucleotides and oligonucleoside phosphorothioates in the 5′-position with the positron-emitting radionuclide ^{18}F ($t_{1/2}$ = 110 min) is described. The label was incorporated by the reaction of succinimido 4-[^{18}F]fluorobenzoate **4** with oligonucleotides (18- and 20-mers) modified in the 5′-position with a hexylamine linker. Oligodeoxynucleotides 5′-GCT.AAG.CGA.TGC.CTC.CGT-3′ (MTCa) and 5′-GAA.CCT.CTG.AGA. GTT.CAT.CT-3′ (CROa) were labelled in 20 ± 3% (MTCa) and 13 ± 3% (CROa) radiochemical yields (non-isolated, decay-corrected and based on **4**). Oligonucleoside phosphorothioates MTCa (S-MTCa) and CROa (S-CROa) were labelled in 9 and 7% isolated radiochemical yield, respectively (decay-corrected and based on **4**). Labelled oligonucleotides and phosphorothioate analogues were separated from their unlabelled counterparts using reversed-phase perfusion chromatography. The molecular mass of a labelled oligonucleotide CROa was determined by ESI-MS after a mixed ^{18}F/^{19}F fluorobenzoate labelling experiment and corresponded with the expected structure.

Positron emission tomography (PET) is a technique that allows the visualisation and quantification of biological functions such as blood flow, receptor-binding, enzymatic function, cell proliferation or energy metabolism *in vivo* by the use of appropriate compounds labelled with positron-emitting radionuclides. Commonly used radionuclides are ^{15}O, ^{13}N, ^{11}C and ^{18}F, with half-lives of 2, 10, 20 and 110 min, respectively.[1,2] A new and interesting area of research in the PET field is the use of antisense oligodeoxynucleotides and their analogues labelled with positron-emitting radionuclides for pharmacokinetic studies and possibly the imaging of *in vitro* and *in vivo in situ* hybridisation.[3,4] These new labelled probes are interesting for use in PET applications because of their high degree of specificity in base pairing to the complementary mRNA sequence and their potential to access biological parameters not previously available because of the lack of appropriate ligands. Oligodeoxynucleotides and analogues labelled with positron emitters might also be interesting research tools for *in vitro* hybridisation applications, due possibly to increased sensitivity related to high specific radioactivity, e.g., of ^{18}F.

The interest in antisense oligodeoxynucleotides has grown as their use has developed from analytical probes in molecular biology to potential therapeutic agents.[5,6] A number of oligonucleotide analogues have been synthesised and investigated, with the aim of increasing the stability and distribution of this type of compound *in vivo*. An important group of analogues have been modified in the phosphate backbone, for example phosphorothioates, phosphorodithioates and methyl phosphonates.[7] Oligodeoxynucleotides (more commonly referred to as oligonucleotides) have also been modified with a wide range of conjugate groups, such as fluorescent, intercalating, cross-linking and chain-cleaving compounds that improve detection, hybridisation or cell uptake.[8] These properties are often introduced by the reaction between a nucleophilic linker attached to the oligonucleotide and an electrophile in the conjugate compound. This is an attractive approach for the labelling of oligonucleotides with short-lived positron emitters, since the same labelling protocol can be used regardless of the sequence and backbone modifications, and the labelling reaction can be performed on already synthesised oligonucleotides. Primary amines or thiols of different alkyl chain lengths are commonly used as the nucleophilic linkers.[9–12] The linkers can be introduced either in the 3′- or 5′-position of the oligonucleotide, and are incorporated during the oligonucleotide synthesis by the use of commercially available reagents. Common electrophiles in the reaction with alkylamine func-

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tionalised oligonucleotides are *N*-hydroxysuccinimide esters and isothiocyanates. These electrophiles are also often used for modifications and radioactive labelling of proteins.[13]

As we have reported, oligonucleotides can be labelled with ^{18}F by using 4-([^{18}F]fluoromethyl)phenyl isothiocyanate.[14] In this study, we investigated labelling of oligonucleotides using a chemically more stable labelled precursor than 4-([^{18}F]fluoromethyl)phenyl isothiocyanate. For this purpose, we used succinimido 4-[^{18}F]fluorobenzoate,[15,16] as the electrophilic ^{18}F-labelled reagent for labelling of the 5′- hexylamine modified oligonucleotides (Fig. 1, Scheme 1). The same reagent was also applied for labelling of oligonucleoside phosphorothioates (commonly referred to as phosphorothioate oligonucleotides).

Experimental

General. All chemicals used were commercially available and used without further purification, unless otherwise indicated. Anhydrous acetonitrile and DMSO were purchased (Aldrich Sure/Seal™) and used as received. Succinimido 4-fluorobenzoate was synthesised by a literature procedure.[16] Spectral data of succinimido 4-fluorobenzoate were in accordance with previously reported data. ^1H NMR (300 MHz) and ^{13}C NMR spectra (75.4 MHz) were recorded on a Varian XL-300 spectrometer, with chloroform-d_1 as the solvent. Tetramethylsilane or chloroform was used as an internal standard. Thin-layer chromatography (TLC) was performed on Alugram Sil G/UV$_{254}$ silica plates (Macherey-Nagel, Düren, Germany). Ultraviolet (UV) absorbance was visualised using short- and long-wave ultraviolet light. Radioactivity on TLC-plates was measured by storage-phosphor autoradiography using a Molecular Dynamics Phosphor Imager® (Sunnyvale, CA, USA). Solid phase extraction (SPE) columns used were C18 Supelclean LC-18 SPE tubes, 500 mg, supplied by Supelco (Bellefonte, PA, USA), Silica Isolute SPE columns, 500 mg, supplied by Sorbent (Hengoed, UK) and Silica Chromabond SPE columns, 100 mg, supplied by Macherey-Nagel. Quantities of oligonucleotides are expressed in OD_{260} units. 1.0 OD_{260} unit equals that amount of oligonucleotide which, when dissolved in 1 ml of H_2O, has an absorbance of 1.0 at 260 nm. The amounts of oligonucleotides was determined using the conversion factor 33 μg oligonucleotide/1.0 OD_{260}.[17] Electrospray ionisation mass spectroscopy ESI-MS was performed using a Fisons Platform (Micromass, Manchester, UK). Data were acquired in the negative ionisation mode by scanning from *m/z* 500 to *m/z* 1100. Samples were dissolved in 2.5 mM imidazole and 2.5 mM piperidine in aqueous 50% isopropyl alcohol.[18] The Maximum Entropy program (part of Mass Lynx Micromass software for Windows NT Version 2.2) was used to reconstitute the true molecular mass spectrum from a multiply charged ion mass spectrum obtained by ESI-MS.

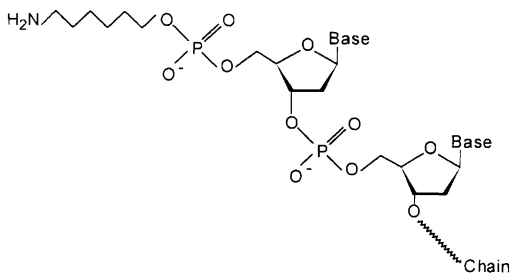
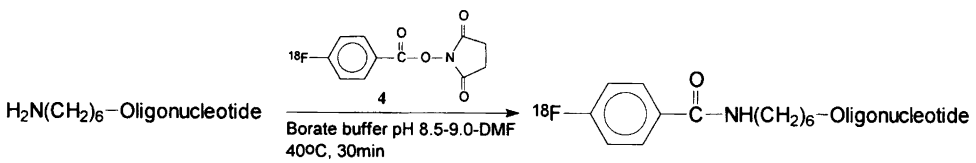


Fig. 1. Oligonucleotide modified in the 5′-position with hexylamine. In oligonucleoside phosphorothioates, one of the oxygen atoms at the phosphorus is replaced by a sulfur atom. These structures are abbreviated $H_2N(CH_2)_6$-oligonucleotide in Scheme 1.

Liquid chromatography. Analytical liquid chromatography (LC) separations were performed using a Beckman (Fullerton, CA, USA) System (a 126 pump and a 166 UV detector, with a β^+-flow detector in series). Data collection was performed using the Beckman System Gold Chromatography Software Package. Columns used were: I, TSK Gel Oligo DNA RP 300 Å high pressure liquid chromatography (HPLC) column (Tosohaas, Montgomeryville, PA, USA) 200 × 4.6 mm ID, 5 μm; II, Ultrasphere ODS C-18 HPLC column (Beckman) 250 × 4.6 mm ID, 5 μm; III, fast desalting HR 10/10 fast protein liquid chromatography (FPLC) gel filtration column (Pharmacia Biotech, Uppsala, Sweden); IV, Poros R2/H perfusion chromatography reversed-phase column (Per Septive Biosystems, Framingham, MA, USA) 100 × 2.1 mm ID, 10 μm. The mobile phase systems used were as follows: system A, flow 0.7 ml min^{-1}, *a* = 95% 0.10 M NH_4OAc (pH unadjusted)-5% MeCN *b* = 90% MeCN–10% 0.10 M NH_4OAc (pH unadjusted), linear gradient 5–95% b 0–10 min, 95% b 10–12 min, linear gradient 95–5% b 12–16 min; system B, flow 1.5 ml min^{-1}, *a* = 0.10 M NH_4OAc (pH unadjusted), *b* = MeCN, 5% b 0–17 min, linear gradient 5–100% b 17–25 min, linear gradient 100–5% b 25–30 min; system C, flow 2.0 ml min^{-1},



Scheme 1.

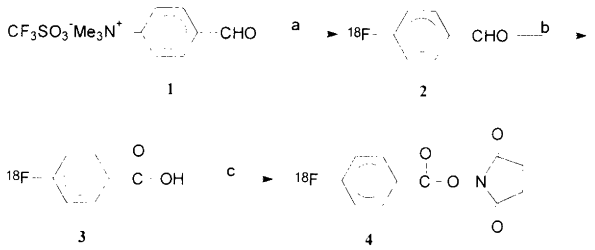
50 mM NaH_2PO_4–Na_2HPO_4 0.16 M NaCl pH 7.3; system D, flow 1.5 ml min^{-1}, a = 50 mM triethylammonium acetate (TEAA), pH 7.0, b = MeCN, 0% b 0–2 min, linear gradient 0–15% b 2–22 min, 15% b 22–28 min, linear gradient 15–60% b 28–31 min, linear gradient 60–0% b 31–34 min; system E, flow 1.5 ml min^{-1}, a = 50 mM TEAA, pH 7.0, b = MeCN, 0% b 0–2 min, linear gradient 0–10% b 2–22 min, 10% b 22–28 min, linear gradient 10–60% b 28–31 min, linear gradient 60–0% b 31–34 min.

Oligonucleotides. Oligonucleotides 5′-GCT,AAG,CGA, TGC,CTC,CGT-3′ (MTCa) and 5′-GAA,CCT,CTG, AGA,GTT,CAT,CT-3′ (CROa) were synthesised on a 1 μmol scale using the phosphoramidite method,[19–21] on an Oligo 1000 DNA synthesizer (Beckman) with β-cyanoethyl *N,N*-diisopropyl-(CED)-phosphoramidites (Beckman). The alkylamine was introduced in the 5′-position of the oligonucleotide as the last step in the synthesis cycle by standard procedures using Aminolink 2™ obtained from Applied Biosystems (Foster City, CA, USA).[22] The crude oligonucleotides were purified by gel filtration (NAP-5 columns, Pharmacia Biotech) followed by precipitation from a 3 M HOAc–NaAc pH 4.8 buffer using cold ethanol.[23,24] Samples of synthesised oligonucleotides were analysed by LC and random samples also by ESI-MS for characterisation.

Oligonucleoside phosphorothioates MTCa (S-MTCa) and CROa (S-CROa) with a hexylamine linker in the 5′-position were purchased from Scandinavian Gene Synthesis AB (Köping, Sweden).

Radionuclide preparation. Aqueous $[^{18}F]F^-$ was produced via the $^{18}O(p,n)^{18}F$ reaction by irradiation of $[^{18}O]H_2O$ by 17 MeV protons in a high pressure silver target using a Scanditronix MC-17 Cyclotron at the Uppsala University PET Centre. The content of the target was washed out with H_2O–MeCN delivered by a Waters 501 pump into a glass vial. The aqueous $[^{18}F]F^-$ solution was added to Kryptofix® 2.2.2. (10 mg, 26.5 μmol) and K_2CO_3 (1.0 mg, 7.3 μmol), and the water was removed by repeated azeotropic evaporation with acetonitrile at 105 °C. The residual radioactive material was dissolved in DMSO (200 μl).

Preparation of 4-[^{18}F]fluorobenzoic acid 3. Crude **3** was prepared following the published procedure (Scheme 2);[15] resolubilised $[^{18}F]F^-$ showing radioactivity was added to 4-formyl-*N,N,N*-trimethylanilinium trifluoromethanesulfonate **1** (2.0 mg, 6.4 μmol) and heated at 130 °C for 10–15 min, diluted with water and the product purified on a C18 solid phase extraction (SPE) column and on a 100 mg silica SPE column eluting with dichloromethane. To the purified 4-$[^{18}F]$fluorbenzaldehyde **2** in an open glass vial was added $KMnO_4$ solution (250 μl, 5% in 1 M NaOH) and the mixture was heated at 130 °C for 5–7 min, during which time the dichloromethane evaporated off. The vial was



Scheme 2. a, $[^{18}F]$KF, Kryptofix 2.2.2., DMSO, 130 °C; b, (i) NaOH, $KMnO_4$, 130 °C; (ii) $Na_2S_2O_5$, HCl; c, DSC, pyridine, MeCN, 150 °C.

cooled in an ice bath and sodium bisulfite was added (100 mg), followed by conc. HCl (150–200 μl).

Crude **3** was diluted with 5 ml water and loaded onto an activated (5 ml MeOH followed by 5 ml water) C18 SPE column. The column was washed with water (5+2+2 ml). The product was eluted with ethyl acetate (6 ml) into a vial containing Na_2SO_4. The dried organic phase was passed through a 500 mg silica SPE column and the solvent was removed on a rotary evaporator. The aldehyde **2** was isolated in 57–80% radiochemical yield (decay-corrected and based on resolubilised $[^{18}F]F^-$) and >99% radiochemical purity. The acid **3** was isolated in 55–60% radiochemical yield and >99% radiochemical purity (decay-corrected and based on **2**). The identities of compounds **2** and **3** were assessed by analytical LC (Table 1) by coelution with 4-fluorobenzaldehyde and 4-fluorobenzoic acid reference compounds, respectively.

Preparation of succinimido 4-[^{18}F]fluorobenzoate 4. The preparation of crude **4** was performed as previously reported;[16] **3** was dissolved in acetonitrile (200 μl) and solutions of disuccinimido carbonate (DSC) (30 μl, 0.1 M in MeCN, 3.0 μmol) and pyridine (30 μl, 0.1 M in MeCN, 3.0 μmol) were added. The mixture was transferred to a small glass vial which was tightly capped and heated at 150 °C for 5 min. The purification of **4** was performed by using a C18 SPE column as follows: the crude product was diluted with water (5 ml) and loaded onto an activated (5 ml MeCN followed by 5 ml water) C18 SPE column; the column was washed with water (5 ml) and MeCN–water 25:75 (2×4 ml); compound **4** was eluted with MeCN (3 ml). The ester **4** was prepared in 9–32% radiochemical yield (decay-corrected and based on resolubilised $[^{18}F]F^-$) within 100 min synthesis time and in >99% radiochemical purity. The identity of **4** was assessed by analytical LC (Table 1) by coelution with the succinimido 4-fluorobenzoate reference compound.

Oligonucleotide labelling. The solvent was removed from **4** (prepared as described above) by using a rotary evaporator or in a stream of nitrogen gas. The oligonucleotide labelling was performed by dissolving the residue containing **4** in *N,N*-dimethylformamide (DMF) (20 μl)

Table 1. Retention times (t_R) of labelled oligonucleotides synthesised from **4**.

Entry	Column	Eluent system	t_R for oligonucleotides/min		t_R for **4**/min	t_R for **3**/min
1	I	A	6.0–6.2[a]		10.3–10.6	4.1–4.2
2	II	B	21.1–21.4[a]		24.5–24.7	6.9–7.1
3	III	C	1.3[a]		3–6[b]	3–6[b]
4	IV	D	25.4[c]	22.1–22.8[d]	31.5–32.0	1.2
5	IV	D	24.3[e]	21.5–21.8[f]	31.5–32.0	1.2
6	IV	E	21.8[g]	18.2–18.3[h]	30.4–32.0	1.2

[a] t_R for labelled and unlabelled oligonucleotides MTCa and CROa (not resolved). [b] The low molecular weight compounds eluted at 3–6 min. [c] t_R for ^{18}F-S-CROa. [d] t_R for S-CROa. [e] t_R for ^{18}F-S-MTCa. [f] t_R for S-MTCa. [g] t_R for ^{18}F-CROa. [h] t_R for CROa.

followed by addition of the oligonucleotide (5–40 OD units, 26–210 nmol) or oligonucleoside phosphorothioates (140 nmol) in 0.05 M pH 8.5 borate buffer (80 µl). The reactions were carried out at 40 °C for 30 min. The reaction mixture was diluted with 100 µl 50 mM TEAA before analysis/isolation by perfusion chromatography (LC system D or E, column IV). Radiochemical yields were determined either by analysis of the reaction mixture or as isolated yields using reversed-phase perfusion chromatography, FPLC gel filtration[25] or reversed-phase HPLC. The retention times for the various labelled oligonucleotides ^{18}F-S-CROa, ^{18}F-S-MTCa, ^{18}F-CROa and ^{18}F-MTCa and the liquid chromatography systems used are presented in Table 1.

Oligonucleotide labelling with **4** *and succinimido 4-fluorobenzoate.* The labelling reaction was performed as described above except that to residual **4** a solution of succinimido 4-fluorobenzoate in DMF (20 µl, 0.6 mg/100 µl DMF, 500 nmol) was added, followed by 10 OD units (≈52 nmol) CROa oligonucleotide in borate buffer. The crude product was purified using LC system E and column IV. Fractions eluting after the unlabelled oligonucleotide were desalted by gel filtration using NAP-5 columns (Pharmacia Biotech) followed by EtOH precipitation from a 3 M pH 4.8 HOAc–NaAc buffer. The samples were analysed by mass spectroscopy as described above. The product eluted at 21.8 min; MS (ESI$^-$) *m/z* 637.9 $[M-10H]^{10-}$; 708.8 $[M-9H]^{9-}$; 797.6 $[M-8H]^{8-}$; 911.4 $[M-7H]^{7-}$.

Results and discussion

The aim of this work was to develop a procedure for oligonucleotide labelling with ^{18}F. In this method, the ^{18}F-label was incorporated in the first synthetic step by a nucleophilic aromatic substitution reaction on an activated aromatic ring using $[^{18}F]F^-$ (Scheme 2). The activated ester **4** was synthesised using a procedure similar to previously reported methods. The reported procedure was followed until the preparation of crude acid **3** was obtained (Scheme 2). The solid phase extraction procedure used for isolating the acid gave more reproducible results in the esterification of **3**, compared with when the reported liquid–liquid extraction procedure for the purification of **3** was followed.[15,16] The radiochemical yields for isolated **3** were in the range 55–60% and the radiochemical purity was >99%, decay-corrected and based on 4-[^{18}F]fluorobenzaldehyde.

The esterification of **3** to produce **4** was performed with DSC as described.[16] DSC was removed from the reaction mixture by use of a reversed-phase C18 SPE column. Removal of DSC seems to be essential since it may cause dimerisation of alkylamine 5′-functionalized oligonucleotides. Remaining DSC has been reported to cause cross-linking and decreased coupling yields in protein labelling reactions.[19] The labelled precursors had to be purified by HPLC (normal phase) in those studies, since purification using SPE silica columns could not overcome the problems. Isolated **4** was prepared in 9–32% radiochemical yield (decay-corrected and based on $[^{18}F]F^-$) and >99% radiochemical purity within 100 min synthesis time. The specific radioactivity of **4** was determined by HPLC to be about 0.8 GBq µmol^{-1} (from 1.9 GBq aqueous $[^{18}F]F^-$).

Oligonucleotide labelling was performed with **4**, in 100 µl 50 mM borate buffer pH 8.5–DMF 80:20 (Table 2). The radiochemical conjugation yields as a

Table 2. Radiochemical yields of labelled oligonucleotides and oligonucleoside phosphorothioates using **4**.

Entry		Radiochemical yield (%) Analytical	Isolated	Amount of oligonucleotide	n[d]
	Oligonucleotides				
1	MTCa[a]	20±3	—	10 OD_{260} units[c]	3
2	MTCa[b]	22	—	20 OD_{260} units[c]	1
3	CROa[a]	13±3	—	10 OD_{260} units[c]	3
	Oligonucleoside phosphorothioates				
5	S-MTCa[a]	12	9	140 nmol	1
6	S-CROa[a]	8	7	140 nmol	1

[a] Reaction conditions: 100 µl 80:20 50 mM pH 8.5 borate buffer–DMF, T=40 °C, 30 min. [b] Reaction conditions: 100 µl 80:20 50 mM pH 9.0 borate buffer–DMF, T=60 °C, 30 min. [c] 10 OD_{260} units equals approximatly 52 and 57 nmol of MTCa and CROa, respectively, using the conversion factor 33 µg oligonucleotide/1.0 OD_{260} unit.[17] [d] The radiochemical yield is given for n experiments.

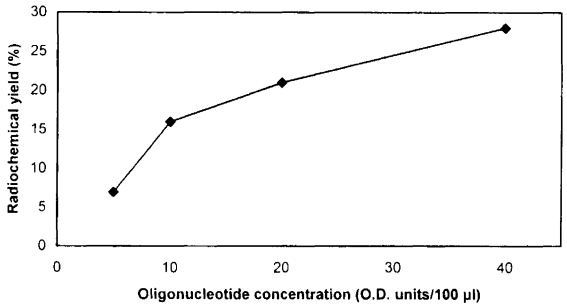


Fig. 2. Radiochemical yields (based on **4**) of ^{18}F-labelled oligonucleotide (CROa). Reaction conditions: 100 µl 50 mM borate buffer pH 8.5–DMF 80:20, 40 °C, 30 min.

function of oligonucleotide concentration were determined using CROa as a model compound. The radiochemical yields varied in the range 7–28% using 5–40 OD units (35–280 nmol) oligonucleotide (Fig. 2). Using more than 20 OD units seemed to overload the column in the purification step. Scaling up the purification step increases the volume of the fraction containing the purified product, which can be inconvenient in subsequent work-up of the purified product. Increasing the pH and temperature did not increase the coupling yield (Table 2, entry 2). Oligonucleoside phosphorothioates S-MTCa and S-CROa were labelled in 9 and 7% isolated radiochemical yield respectively, using **4** (Table 2).

In order to investigate the position of the label in the oligonucleotide, a control experiment was performed, (Fig. 3). Compound **4** was incubated with an oligonucleotide bearing a hexylamine linker in the 5′-position, with an oligonucleotide without a hexylamine linker, and without oligonucleotide (chromatograms 3b, 3c and 3d, respectively, Fig. 3). In chromatograms 3c and 3d, the peak corresponding to labelled oligonucleotide is missing, indicating that the label is most likely attached to the alkylamine linker.

Labelled oligonucleotides and phosphorothioate analogues were separated from their unlabelled counterparts using a perfusion chromatography reversed-phase column (Table 1). The products were characterised as >99% high molecular weight species using an FPLC gel filtration column.

The labelled oligonucleotide CROa was characterised by ESI-MS after a mixed ^{18}F/^{19}F-fluorobenzoate labelling synthesis. The product was isolated and desalted before the mass spectrum was acquired (Fig. 4). A molecular mass of 6388.8 ± 0.3 Da was determined after reconstitution to the true molecular mass spectrum (expected mass 6389.1 Da).

To conclude, a method has been developed for labelling of oligonucleotides and oligonucleoside phosphorothioates by the use of succinimido 4-[^{18}F]fluorobenzoate **4**, including isolation and characterisation of the labelled oligonucleotides and their phosphorothioate analogues. Radiochemical yields in the range 7–23% (from **4**) were obtained in the oligonucleotide labelling step. This

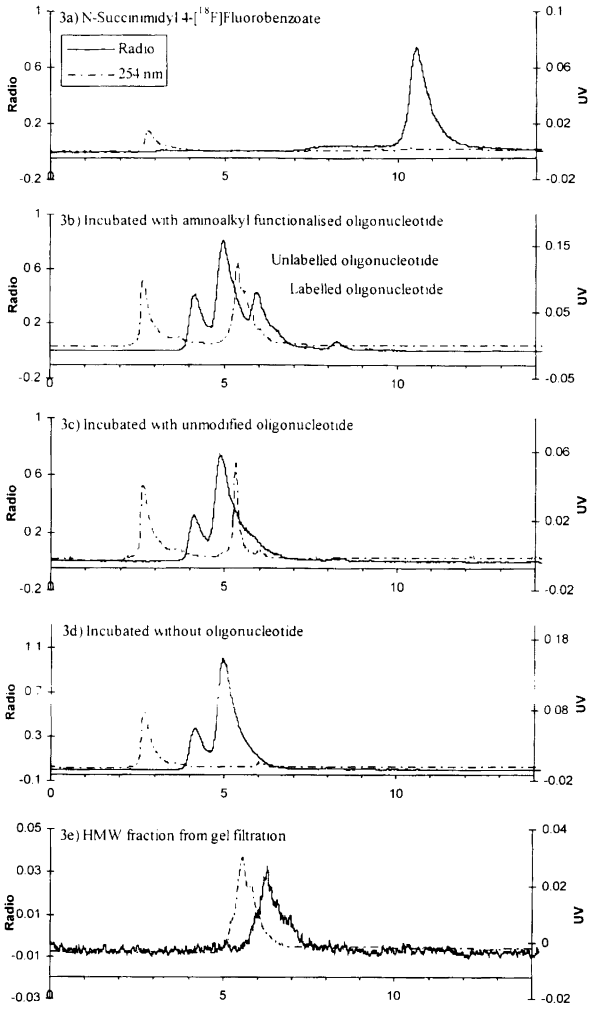


Fig. 3. Analytical HPLC chromatograms of oligonucleotide (MTCa) labelling experiment, 0–14 min, column I, mobile phase method A. Comments: (3b) the corresponding UV-peak of the labelled oligonucletide is not visible due to a combination of insufficient resolution and the relatively small amount of labelled compared with unlabelled oligonucleotide. (3e) HMW = high molecular weight.

method is preferred over the published method using an isothiocyanate reagent,[14] since in this case, no dehalogenation of the labelled oligonucleotides was observed using the analytical methods described. Two major drawbacks of this method are the long multi-step synthesis for the preparation of **4** and the relatively low yields in the conjugation step with the oligonucleotides. An alternative and promising method for the synthesis of **4** has been published recently with radiochemical yields of **4** reported to be 55 ± 10% within 35 min synthesis time.[26] The oligonucleotide labelling step will be further investigated and experiments are in progress to evaluate the properties of the labelled oligonucleotides and analogues.

Acknowledgments. This work was supported financially by the Swedish Natural Science Research Council, grant K 3463, the Swedish Cancer Society and Japan Science Technology Corporation.

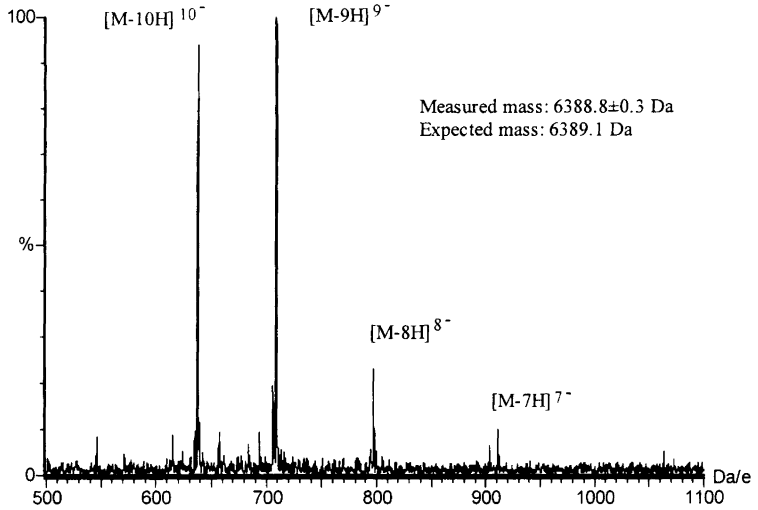


Fig. 4. Multiply charged ion mass spectrum of the ^{19}F-fluorobenzoate labelled oligonucleotide CROa. The $^{19}F/^{18}F$-fluorobenzoate labelled oligonucleotide was separated from the unlabelled oligonucleotide (Table 1) using LC system E and column IV, after a mixed $^{19}F/^{18}F$-fluorobenzoate labelling synthesis. The measured molecular mass (6388.8 ± 0.3 Da) corresponds to the expected structure (expected mass 6389.1 Da).

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25. When performing gel filtration on small amounts ($\leq$1 OD unit) of oligonucleotide labelling reaction mixture using NAP-5 or PD-10 columns (Pharmacia Biotech) eluted with water, a loss of separation between the low molecular weight (LMW) and high molecular weight (HMW) compounds was observed.
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