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Characterization of antisense oligonucleotide–peptide conjugates with negative ionization electrospray mass spectrometry and liquid chromatography–mass spectrometry

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Abstract

Covalent post-synthesis or solid-phase conjugation of peptides to oligonucleotides has been reported as a possible method of delivering antisense oligonucleotides into cells. While synthesis strategies for preparing these conjugates have been widely addressed, few detailed reports on their structural characterization have been published. This paper discusses the negative ion electrospray ionization mass spectrometric (ESI-MS) and liquid chromatography–mass spectrometric (LC–MS) analysis of various peptide–oligonucleotide conjugates ranging from small T_6-nucleopeptides to large peptide–oligonucleotide phosphorothioate conjugates and ribozyme–peptide hybrids (3–13 kDa). Molecular weight determination with mass errors of 0.1–3.1 amu were conducted, employing on-line IP-RP-HPLC and high m/z range mode to facilitate the analysis of large compounds and difficult modifications.
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1. Introduction

Antisense oligonucleotides are new potential drugs showing promise for the treatment of viral infections and cancer [1,2]. Their systemic therapeutic use is, however, hindered by their poor cellular uptake and inability to reach the nucleus. Oligonucleotides have been found to be taken into cells by endocytosis, after which they remain entrapped in endosomal compartments [3]. Several strategies have been introduced to overcome this problem, including the attachment of lipophilic or cell receptor-binding molecules to oligonucleotides [4]. Peptides possessing various beneficial properties have been seen as a possible method of delivery [5]. Several studies have employed hydrophobic peptide sequences known to penetrate cellular membranes [6,7], nuclear localization sequences [8], or both of them combined [5,9]. Many

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Table 1
Oligonucleotide sequences

Name	Sequence
SAS1	5′-TGG CGT CTT CCA TTT-3′
LSAS1	5′-TTT TGG CGT CTT CCA TTT TAC CAA C-3′
SC1	5′-TTT ACC TTC TGC GGT-3′
SC2	5′-ACC GCA GAA GGT AAA-3′
Rz[a]	5′-$g_s c_s a_s$ $g_s$ug gcc gaa agg Cga gUg aGG uCu agc uca B-3′

SAS1 and LSAS1, antisense inhibitors of firefly luciferase mRNA; SC1, reversed control; SC2, sense control; Rz, ribozyme "zinzyme".

[a] Lower case, 2′-OMe; C, 2′amino-C; U, c-allyl-U; G, ribo G; B, inverted abasic; s, phosphorothioate linkages.

of the conjugates have indeed exhibited enhanced cellular delivery and accumulation in the nucleus [6,9]. In addition, 3′-conjugation seems to stabilize phosphodiester oligonucleotides against nucleases [10], and no adverse effect on duplex formation has been observed [8,11]. The use of noncovalently complexed peptide–oligonucleotide hybrids has been reported [6,10], but the main interest has lately been on covalently attached conjugates. They can be synthesized either by post-synthetic coupling of pre-purified peptides and oligonucleotides [9,12] or by stepwise solid phase synthesis without intermediate purifications [11,13,14], the latter being more convenient for routine applications because of its speed and easiness.

Electrospray ionization mass spectrometric (ESI-MS) analysis of peptides and, increasingly, of oligonucleotides can be considered a routine method. A number of peptide–oligonucleotide conjugates have been characterized by MALDI-MS [15,16], ion spray MS [11] or ESI-MS [7,9,13,14,17]. However, very few detailed articles can be found on the mass spectrometry of peptide–oligonucleotide conjugates. The successful ESI-MS of peptide–oligothymidylates has been reported on a triple–quadrupole system, including attempts to sequence these conjugates by tandem-MS [18].

In this study, negative ionization ion trap ESI-MS and LC–MS were used to characterize several peptide–oligonucleotide conjugates synthesized either by post-synthetic conjugation (convergent synthesis) or direct solid-phase assembly (stepwise synthesis).

2. Experimental

2.1. Reagents and chemicals

DNA synthesis reagents were obtained from Glen Research, except 2,6-lutidine (99%) and *N*-methylimidazole (99+%, redistilled) which were from Aldrich. The Fmoc-L-amino acids and resins for peptide synthesis were purchased from Bachem and Novabiochem. 2,2′-Bis-dipyridyl disulfide (98%, Aldrithiol™) and threo-1,4-dimercapto-2,3-butandiol (1,4-dithio-D,L-threitol; 98%) were from

Table 2
Peptide sequences

Name	Sequence
MPMc	H–AAVALLPAVLLALLAPC–NH_2
MPMn	HS–$(CH_2)_2$–CO–AAVALLPAVLLALLAP–NH_2
NLSc	H–VQRKRQKLMPC–OH
NLSn	HS–$(CH_2)_2$–CO–VQRKRQKLMP–OH
MPM-NLSc	H–AAVALLPAVLLALLAPVQRKRQKLMPC–OH
MPM-NLSn	HS–$(CH_2)_2$–CO–AAVALLPAVLLALLAPVQRKRQKLMP–OH
TAT	H–GRKKRRQRRRPPQC–OH
ANT	H–RQIKIWFQNRRMKWKKC–OH

MPM, membrane permeable motif (hydrophobic region of a signal peptide sequence of the Kaposi fibroblast growth factor); NLS, nuclear localization sequence of transcription nuclear factor *kB*; TAT, truncated HIV-1 Tat protein basic domain; ANT, 43–58 homeodomain of p*Antennapedia*; c, thiol group at C-terminus; n, thiol group at N-terminus.

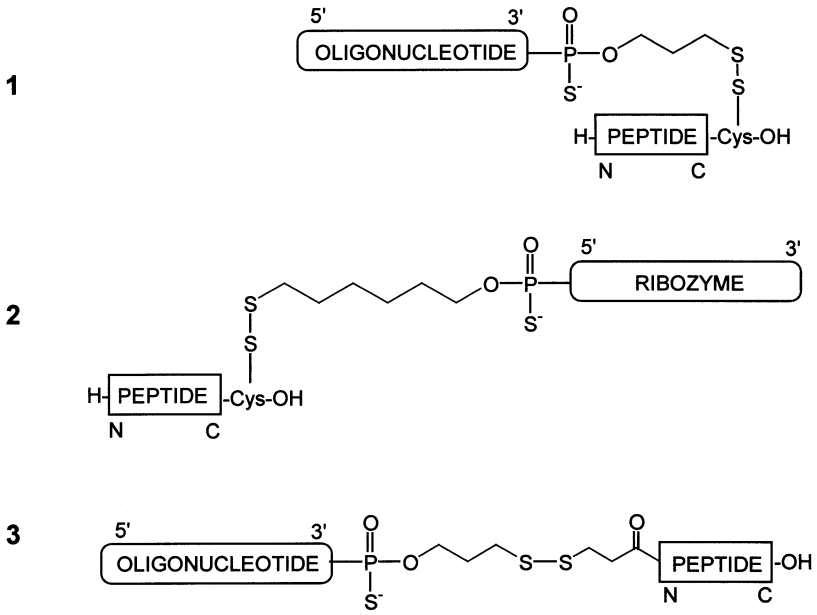


Fig. 1. Structures of the conjugates prepared by convergent synthesis: linked via (1) peptide C-terminus and oligonucleotide 3′-terminus, (2) peptide C-terminus and ribozyme 5′-terminus or (3) peptide N-terminus and oligonucleotide 3′-terminus.

Aldrich. Acetonitrile (ACN) and other solvents were HPLC grade.

LC–MS eluents containing triethylammonium acetate (TEAA) and dimethylhexylammonium formate (DMHA-F) were prepared by adjusting the pH of 25 mM triethylamine (TEA; puriss. p.a., for HPLC, >99.5%, Fluka) to 7 with acetic acid or by adjusting the pH of 20 mM *N*,*N*-dimethylhexylamine (DMHA; 98%, Aldrich) to 7 with formic acid. The pH was adjusted before adding ACN to any eluents. The eluents containing 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP; puriss. p.a., for GC, ≥99.8%, Fluka) were prepared as described earlier [19].

2.2. Synthesis

2.2.1. Convergent synthesis

The oligonucleotides were synthesized on an Applied Biosystems 392 DNA/RNA synthesizer using standard methods, as described earlier [20], introducing a masked mercapto function at the 3′-

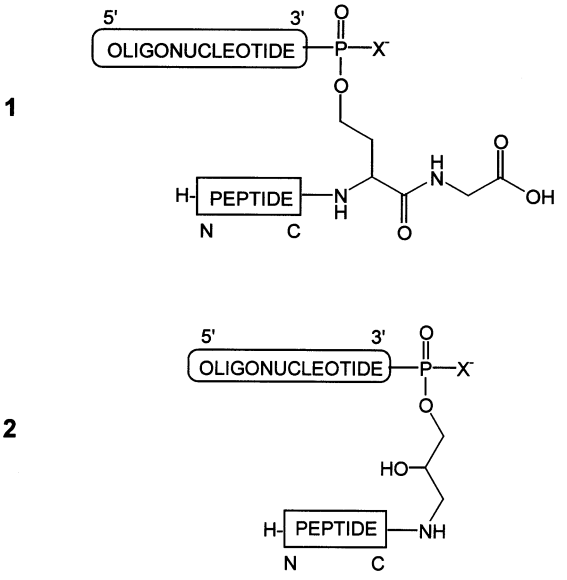


Fig. 2. Structures of the conjugates prepared by stepwise synthesis: (1) HseGly-linker, (2) APD-linker. X = O (phosphodiester T_6) or S (phosphorothioate SAS1).

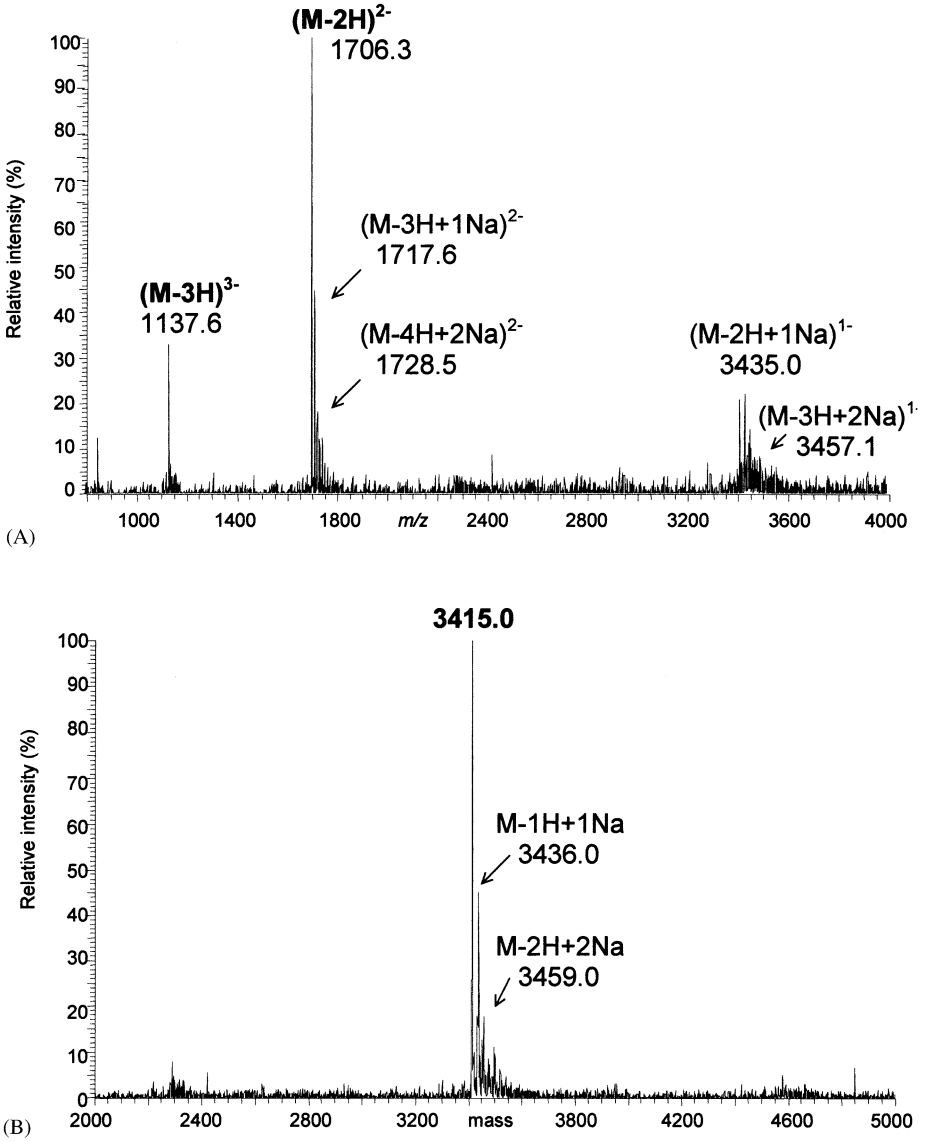


Fig. 3. Full scan (A) and reconstructed (B) negative ion ESI mass spectra of conjugate T_6-(APD)-MPM (calculated MW 3412.2). Direct injection in 25 mM TEA/80% ACN.

terminus of each oligonucleotide. The sequences included 15- and 25-mer antisense inhibitors of firefly luciferase mRNA and 15-mer reversed and sense control sequences (Table 1). The 15-mer antisense oligonucleotide was also fluorescent labeled at the 5′-terminus by using fluorescein phosphoramidite during synthesis. In addition to these, a 34-mer ribozyme bearing a C6-thiol linker at the 5′-terminus, synthesized at RPI (Ribozyme Pharmaceuticals, Inc.), was used for conjugation.

The peptides were synthesized on a Milligen 9050 peptide synthesizer, using Fmoc/HBTU/TEA chemistry [9]. The peptide sequences ranged from 10 to 27 amino acids and included a K-FGF

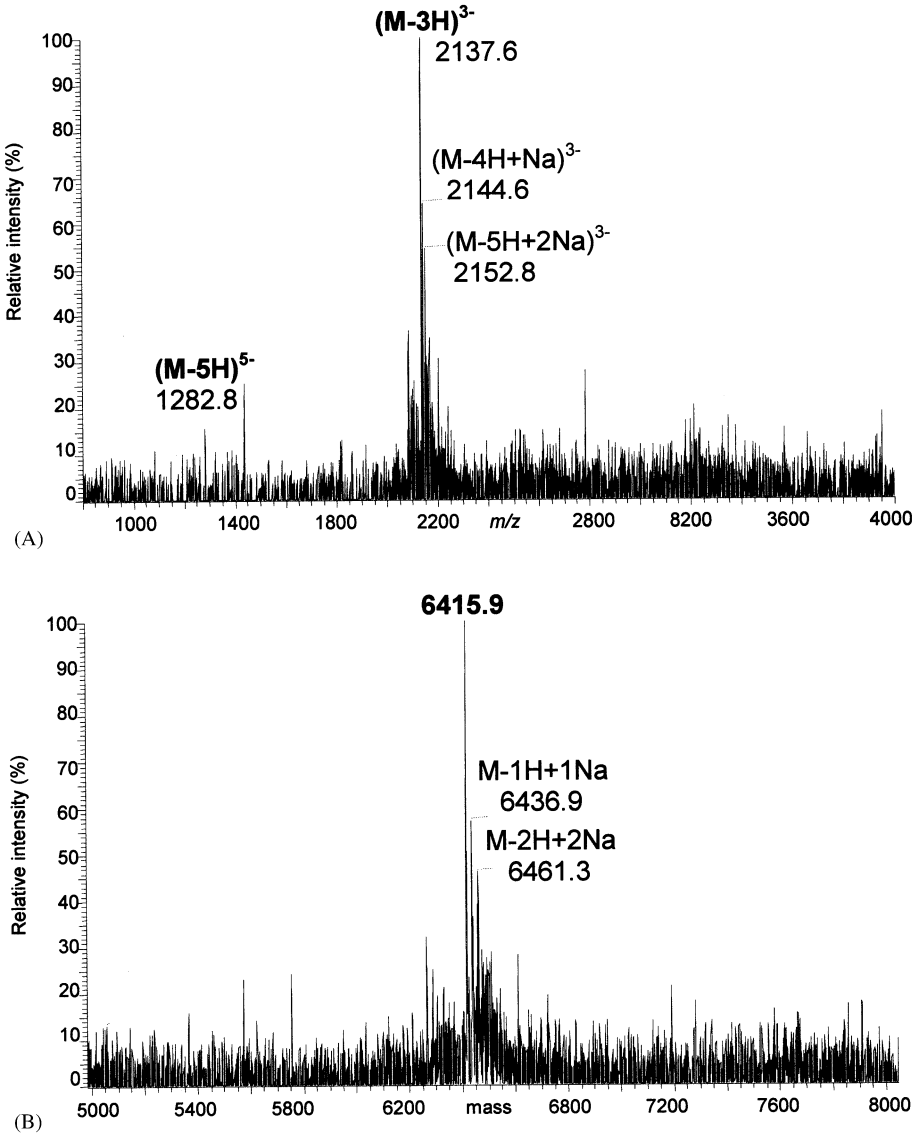


Fig. 4. Full scan (A) and reconstructed (B) negative ion ESI mass spectra of conjugate SAS1-(APD)-MPM (calculated MW 6414.9). Direct injection in 25 mM TEA/80% ACN.

membrane permeable motif, NF-*kB* nuclear localization signal, HIV-1 Tat basic domain and *Antennapedia* homeodomain (Table 2). 3-Tritylthiopropionic acid was attached to the N-termini of some of the peptides directly on the synthesizer.

The peptide–oligonucleotide conjugates were prepared by activating the free thiol group of the peptide with bis-2,2′-dipyridyl disulfide and reacting it with the free thiol group of the oligonucleotide. The masked thiol function of the oligonucleotide was first released by reducing it

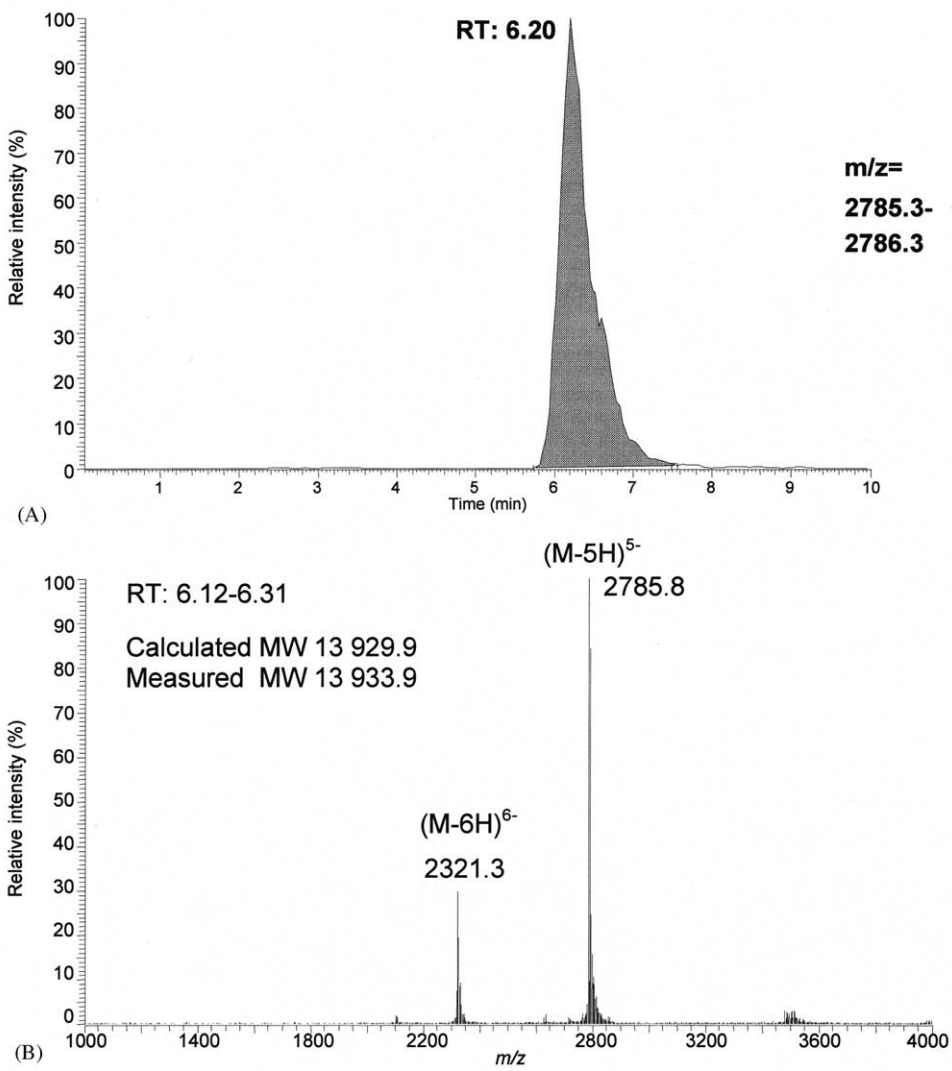


Fig. 5. Selected ion chromatogram (A) and full scan negative ion ESI mass spectrum (B) of ribozyme conjugate Rz-ANT. Injection of 30 pmol, (A), TEAA (pH 7; 25 mM), (B) TEAA (pH 7; 25 mM) in 75% ACN, gradient: 5–100% B, 5+1 min, 100 μl/min, Luna C18 column (50 × 2 mm i.d., Phenomenex).

with dithiothreitol [9]. The general structures of the conjugates are shown in Fig. 1.

2.2.2. Stepwise synthesis

In the stepwise solid-phase synthesis, the peptide MPM (see Table 2) was first assembled with the synthesizer mentioned above, employing standard Fmoc/TBTU/TEA chemistry and a solid support bearing either a homoserine–glycine (HseGly) or 3-aminopropan-1,2-diol (APD) linker [17]. The oligonucleotide part, which was either phosphodiester T_6 or phosphorothioate SAS1 (see Table 1), was then synthesized with the synthesizer mentioned above, cleaved and deprotected using standard protocols. The general structures of the conjugates are shown in Fig. 2.

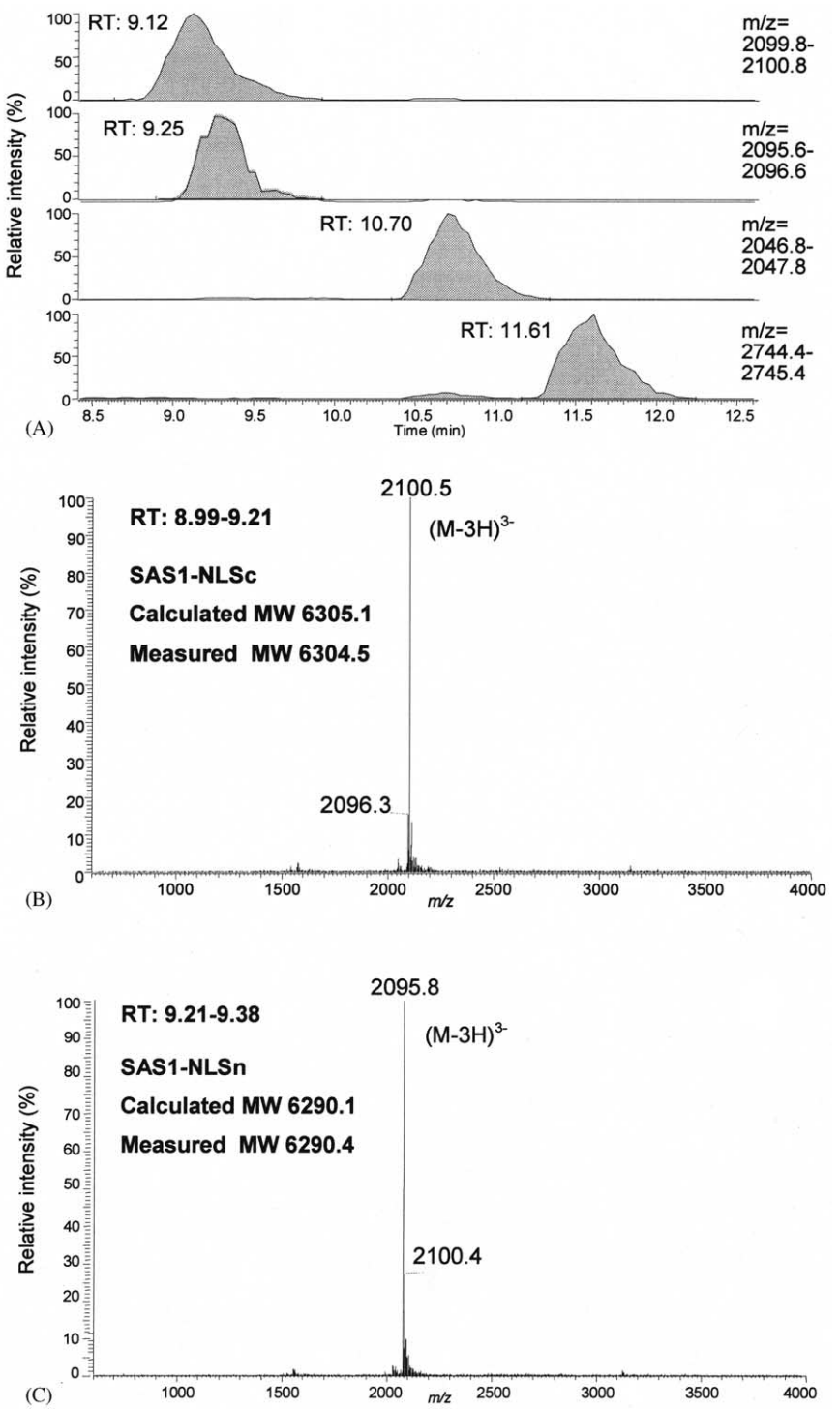
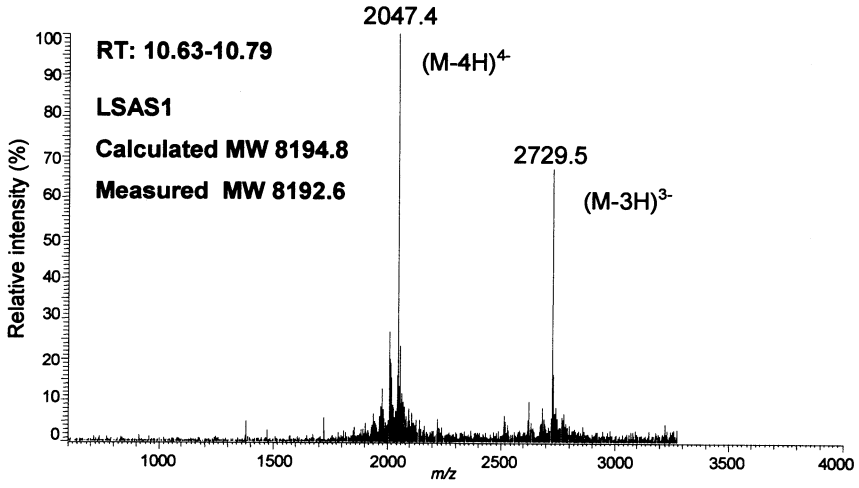
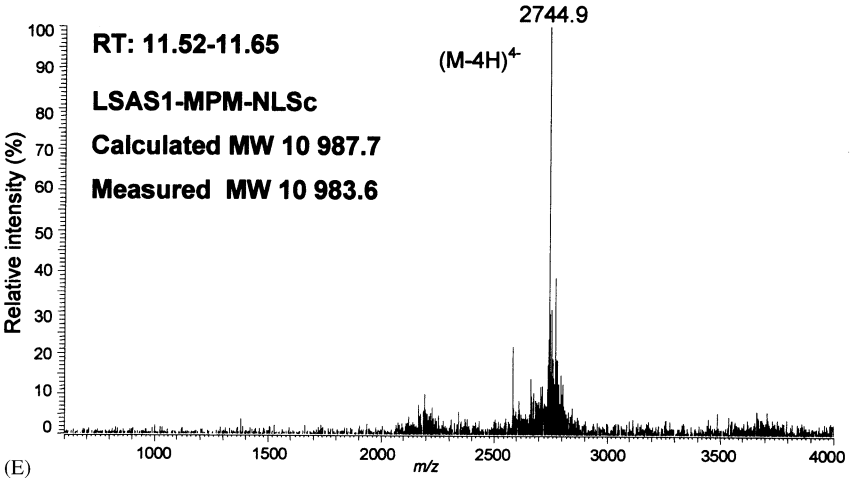


Fig. 6. Selected ion chromatograms (A) and full scan negative ion ESI mass spectra (B–E) of short conjugates SAS1-NLSc and SAS1-NLSn, long oligonucleotide LSAS1 and long conjugate LSAS1-MPM-NLSc, respectively. Injection of 20 pmol each, (A) DMHA-F (pH 7; 20 mM), (B) 90% ACN, gradient: 10–80% B, 20+3 min, 100 μl/min, Genesis C4 column (50 × 2 mm i.d.).



(D)



(E)

Fig. 6 (*Continued*)

2.3. ESI-MS

The conjugates were analyzed on an LCQ quadrupole ion trap ESI mass spectrometer (Finnigan MAT, San Jose, CA) in the negative ion mode. The compounds were diluted to 10–30 μM for direct injection and 1–10 μM for LC–MS analysis. Some of the measurements were done in the high mass-to-charge (m/z) range mode which facilitates the analysis of large compounds by enabling the detection of lower charge states up to m/z 4000.

Most of the conjugates were analyzed using direct 5 μl injections to the mass spectrometer, the eluent consisting of 25 mM TEA in 80% ACN. The eluent flow rate was 5–10 μl/min. The spray needle was set to −3 kV, the nitrogen sheath gas flow rate to 70 and the tube lens offset to −10 V

Table 3
Measured and calculated molecular weights of conjugates

Conjugate	Calculated	Measured	Error (amu)
SAS1-MPMc	6536.4	6533.3	3.1
SAS1-MPMn	6521.4	6519.7	1.7
SAS1-NLSc[a,b]	6305.1	6304.5	0.6
SAS1-NLSn[a,b]	6290.1	6289.3	0.8
SAS1-MPM-NLSc	7803.0	7800.5	2.5
SAS1-MPM-NLSn	7788.0	7787.3	0.7
SAS1-TAT	6740.2	6739.0	1.2
SAS1-ANT[a,b]	7268.0	7268.1	0.1
FL-SAS1-MPMc	7151.5	7151.7	0.2
FL-SAS1-MPMn	7138.5	7137.2	1.3
FL-SAS1-MPM-NLSc	8418.1	8418.0	0.1
FL-SAS1-MPM-NLSn	8405.1	8403.2	1.9
LSAS1-MPM-NLSc[a,b]	10 987.7	10 986.5	1.2
LSAS1-MPM-NLSn	10 974.7	10 973.3	1.4
SC1-MPMc	6536.4	6534.5	1.9
SC1-MPMn	6521.4	6518.6	2.8
SC1-MPM-NLSc	7803.0	7801.8	1.2
SC1-MPM-NLSn	7788.0	7787.7	0.3
SC2-MPMc	6630.5	6627.5	3.0
SC2-MPMn	6615.5	6613.9	1.6
SC2-NLSc	6399.2	6398.5	0.7
SC2-NLSn	6384.2	6383.3	0.9
SC2-MPM-NLSc	7897.1	7896.9	0.2
SC2-MPM-NLSn	7881.5	7880.7	0.8
SC2-TAT	6833.4	6834.8	1.4
Rz-ANT[a,b]	13 929.9	13 933.9	0.4
T_6-HseGly-MPM[a,b,c]	3497.3	3498.0	0.7
T_6-(APD)-MPM[a,b,c]	3412.2	3413.8	1.6
SAS1-HseGly-MPM[a,b,c]	6500.0	6499.5	0.5
SAS1-(APD)-MPM[a,b,c]	6414.9	6415.9	1.0

[a] Measured using the high m/z range mode.
[b] Measured with LC–MS.
[c] Prepared by stepwise solid-phase synthesis.

(−30 V for high m/z range). The stainless-steel inlet capillary was heated to 200 °C, and the capillary voltage was −39 V. The spectra were measured using 200 ms for collection of the ions in the trap, and 5–12 microscans were summed.

The LC–MS measurements were done in similar conditions, using narrowbore LC columns and flow rates of 100–200 μl/min. Typically, a 50 × 2 mm C18 column was used with a gradient of 10–90% ACN or methanol and 15–25 mM TEAA or DMHA-F as the ion-pair reagent.

The mass spectra were deconvoluted, i.e. the molecular weights of the compounds were reconstructed from the spectra, either manually using the formula $z_2 = (m/z_1 + 1)/(m/z_1 - m/z_2)$ or with computer programs (e.g. BIOMASS deconvolution).

3. Results and discussion

As reported earlier, the behavior of oligonucleotide–peptide conjugates in ESI-MS is mainly due to their oligonucleotide portion and negative ionization is suitable for their analysis, especially when the oligonucleotide contributes more to the conjugate mass than does the peptide [18]. As is the case for oligonucleotides, the mass spectra obtained here show a series of multiply deprotonated ions $(M-nH)^{n-}$ as major ions [21]. The charge states range from 2 to 12 lost protons, depending on the size of the molecule, on the MS conditions during analysis and on the eluent used [22]. Some sodium adduct ions are also seen after direct injection ESI-MS of conjugates, although the samples were desalted and the eluent contained organic base. As can be seen from the spectra in Figs. 3 and 4, the degree of cation adduction increases with the length of the oligonucleotide and is more extensive with phosphorothioates than with phosphodiesters [23].

The background noise is, however, significantly reduced by the use of LC–MS. The high m/z range further increases the signal-to-noise (S/N) ratio; it is especially useful when analyzing molecules larger than 10 kDa which often produce uninterpretable spectra below m/z 2000 (Fig. 5). Using LC–MS, a fair resolution of relatively similar compounds can be obtained. As can be seen from Fig. 6(A–C), the almost identical conjugates SAS1-NLSc and SAS1-NLSn are not completely resolved—the chromatogram peaks overlap and peaks corresponding to both compounds are seen on the spectra. Nevertheless, the spectra obtained are more than adequate for simple molecular weight confirmation. The longer oligonucleotide and its conjugate are well resolved (Fig. 6(D–E)). LC–MS eluents used for oligonucleotides contain organic bases such as TEA, tripropylamine (TPA) or diisopropylethylamine (DIPEA), while the acid component of the ion pair reagent is usually acetic acid, formic acid or carbonic acid [24–26]. Some

papers report also the advantages of using HFIP with TEA and a methanol gradient [19,27]. It was found during this study that at least TEAA and DMHA-F with ACN gradients seem to work well for the LC–MS of peptide–oligonucleotide conjugates (Figs. 5 and 6).

Altogether, 30 conjugates were successfully characterized. The measured molecular weights correlated well with the calculated values, the error being 3.1 amu or less in all cases (Table 3). While the ESI-MS of oligonucleotide–peptide conjugates is generally as straight-forward as that of oligonucleotides, some difficult modifications remain that cause problems during analysis. These include conjugates where the peptide part contains a large number of arginine and lysine residues which can bind various salts irreversibly during HPLC purifications, thus severely complicating the spectra, and conjugates assembled by stepwise solid-phase synthesis where no intermediate purifications are used to remove side products, yielding complex mixtures analyzable by HPLC but showing no MS peaks corresponding to the right mass. Hence, some synthesis products were encountered during this work which could not be successfully measured with the current MS methods (data not shown). Fortunately there were very few of these compounds, and the methods described here can be used as an efficient and rapid way to characterize novel peptide–oligonucleotide conjugates in most cases.

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