“Synthesis of oligonucleotide-peptide conjugates for biomedical and technological applications” Aviñó, A., Grijalvo, S., Pérez-Rentero, S., Garibotti, A., Terrazas, M., Eritja, R. Methods in Molecular Biology, “Bioconjugation Protocols 2nd edition”. (S. Mark Ed.) Humana Press, Springer, New York, vol. 751, Part 2, pp 223-238 (2011).
doi: 10.1007/978-1-61779-151-2_14

Synthesis of oligonucleotide-peptide conjugates for biomedical and technological applications

Anna Aviñó, Santiago Grijalvo, Sónia Pérez-Rentero, Alejandra Garibotti, Montserrat Terrazas and Ramon Eritja*

Institute for Research in Biomedicine, IQAC-CSIC, CIBER-BBN Networking Centre on Bioengineering, Biomaterials and Nanomedicine,
Helix Building, Baldiri Reixac 15,
E-08028 Barcelona
SPAIN

*To whom correspondence should be addressed.

Tel: +34 (93) 4039942

FAX: +34 (93) 2045904

E-mail: ramon.eritja@irbbarcelona.org

Abstract

Oligonucleotide-peptide conjugates have attracted considerable interest especially for biomedical uses. In the first part of this chapter, we describe protocols for the stepwise synthesis of oligonucleotides carrying peptide sequences at the 3'-end on a single support. The resulting oligonucleotide-peptide conjugates may be used as exogenous effectors for the specific control of gene expression. In the second part of this chapter, detailed post-synthetic conjugation protocols to introduce peptide sequences into oligonucleotide sequences are also presented.

Key words: DNA, RNA, nucleic acids, RNA interference, antisense, oligonucleotide-peptide conjugates, stepwise synthesis, post-synthetic conjugation.

1. Introduction

Oligonucleotide-peptide conjugates are chimeras of oligonucleotides and peptides that are produced in order to add some of the biological and/or biophysical properties of peptides to oligonucleotides. A peptide sequence may be attached to oligonucleotides either at the 5'- or 3'-terminal ends, or linked to the nucleobases at the internal positions (**Fig. 1**). Most frequently, peptides are linked at the terminal positions of oligonucleotides since their attachment at internal sites can potentially interfere with the base-pairing properties of the nucleobases. Peptide-oligonucleotide conjugates are prepared through distinct conjugation chemistries, which include either stable or cleavable linkages. In the post-synthetic conjugation approach, the two moieties are prepared independently using solid-phase synthesis, and then thiols and maleimido groups are specifically introduced to link the two molecules together (**1**). In the stepwise synthesis approach, oligonucleotide-peptide conjugates are prepared by the addition of protected amino acids and nucleotides during solid-phase synthesis on the same solid support (**2-4**). In this case, the main challenge is to develop an effective protection strategy for peptide synthesis that is compatible with oligonucleotide synthesis. For example, during the final steps of solid-phase peptide synthesis a treatment with acid is usually required, which can lead to partial depurination of DNA oligonucleotides. In the case of the synthesis of oligonucleotide 3'-peptide conjugates, the solution to this issue is the use of *t*-butoxycarbonyl (Boc)-protected amino acids carrying fluorenylmethyl (Fm) or fluorenylmethoxycarbonyl (Fmoc) groups for the protection of the side chains (**2-4**). This unusual Boc/Fmoc strategy, described in several recent reviews (**5-9**), is compatible with oligonucleotide synthesis, and most of the required amino acid derivatives can be obtained from commercial sources.

Herein, we describe protocols for the preparation of oligonucleotide-peptide conjugates developed in our research group at IRB. Specifically, we describe the stepwise synthesis of DNA and RNA molecules covalently linked to peptides that are designed to enhance cellular uptake in antisense (**10**) and RNA interference (**11-12**) experiments. In addition, the synthesis of DNA molecules carrying peptide epitopes by post-synthetic conjugation (**13**) is also discussed.

Figure 1. Illustration of the potential sites within an oligonucleotide that are available for the attachment of peptides.

2. Materials

2.1. Solid-phase synthesis of the peptide moiety

1. Boc-amino acids carrying base-labile groups are standard reagents that are commercially available from a number of suppliers. The protecting groups of lysine and ornithine are either Fmoc or trifluoroacetyl. Glutamic and aspartic acid are protected with the Fm ester. Threonine, tyrosine and serine are protected with the acetyl group. Histidine can be protected with the tosyl group, and cysteine with the Fm or the *t*-butylthio group (7). The remaining amino acids were used without protection. Arginine can be protected with the di-Fmoc protecting group, but this derivative is not commercially available at present (*see* **Note 1**)
2. The coupling agent for peptide synthesis was benzotriazol-1-yl-oxy-tris(pyrrolidino)phosphonium hexafluorophosphate (PyBOP).
3. The solid-phase synthesis support, amino-polyethylene glycol-polystyrene (PEG-PS), was obtained from Perseptive Biosystems.

2.2. Synthesis of oligonucleotides carrying peptide sequences at the 3'-end

1. Oligonucleotide sequences were prepared by solid-phase synthesis using 2-cyanoethyl phosphoramidites as monomers. The phosphoramidites of the natural nucleosides are standard reagents that are commercially available (*see* **Note 2**). The base protection scheme employed during DNA synthesis was as follows: 2'-Deoxyguanosine was protected with either the isobutyryl group or the dimethylaminomethylidene group; and 2'-deoxycytidine and 2'-deoxyadenosine were protected with the benzoyl group. Base protection during RNA synthesis was as follows: Guanosine was protected with the dimethylaminomethylidene group;

cytidine was protected with the acetyl group; and adenosine with the benzoyl group. The 2'-OH protecting group employed for the RNA monomers was the *t*-butyldimethylsilyl (TBDMS) group.

2. Ancillary reagents used during oligonucleotide synthesis included the following: 0.4 M 1*H*-tetrazole in acetonitrile (ACN) (activation); 3% trichloroacetic acid in dichloromethane (DCM) (deprotection), acetic anhydride / pyridine / tetrahydrofuran (1:1:8, v/v/v) (capping A), 10 % (v/v) *N*-methylimidazole in tetrahydrofuran (capping B), 0.01 M iodine in tetrahydrofuran / pyridine / water (7:2:1, v/v/v) (oxidation). All of the preceding solutions can be obtained from the same companies that provide phosphoramidites (*see Note 2*).
3. 6-Aminohexanol.
4. Succinic anhydride.
5. *N,N*-dimethylaminopyridine (DMAP).
6. 4-hydroxybutyric acid.
7. Preparative high-performance liquid chromatography (HPLC) system equipped with a Nucleosil® 120-10 C₁₈ column (250 x 4 mm) (Macherey-Nagel, Inc.).
8. Analytical HPLC system equipped with an XBridge™ OST C₁₈ column (2.5 µm, 4.6 x 50 mm) (Waters).
9. Solvent A for HPLC: 5% acetonitrile in 100 mM aqueous triethylammonium acetate (TEAA), pH 6.5.
10. Solvent B for HPLC: 70% acetonitrile in 100 mM aqueous TEAA, pH 6.5.
11. Voyager-DETMRP mass spectrometer equipped with a nitrogen laser (337 nm) (PerSeptive Biosystems).
12. Polyacrylamide gel electrophoresis system (Hoefer Scientific).
13. Stains-All nucleic acid and protein staining solution (Sigma-Aldrich) was prepared at 0.01% (w/v) in formamide / water (1/1.2 v/v).

2.3. Post-synthetic conjugation of peptides to oligonucleotides carrying thiol groups

1. The following reagents were used for the introduction of thiol groups into oligonucleotides and were obtained from commercial sources: At the 3'-end: 3'-thiol-modifier C3 S-S CPG; and at the 5'-end: 5'-thiol modifier C6 S-S phosphoramidite (*see Note 2 and Fig. 2*). A 2'-deoxycytidine derivative was used for the introduction of a thiol group at internal nucleobase positions (**Fig. 2**), as described in ref. (**13**).
2. For the introduction of maleimido groups at the N-terminal position of peptides, 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS) was used.
3. Rink-Amide polystyrene solid support was used for the synthesis of peptides by the Fmoc / *t*-butyl strategy.

- Standard Fmoc-amino acids protected with *t*-butyl groups were used; and HATU, (O-7-azabenzotriazole-1-yl)-*N,N,N',N'*-tetraethyluronium hexafluorophosphate, was used as a coupling agent.
- Illustra NAPTM-10 desalting column prepacked with Sephadex G-25 (GE Healthcare).

Figure 2. Reagents used for the synthesis of modified oligonucleotides containing thiol groups

3. Methods

There is currently great interest in the use of oligonucleotide-peptide conjugates for a variety of biomedical applications. As discussed in the introduction to this chapter, there are two major approaches that may be used to prepare these types of compounds (*see* **Note 3**). In the post-synthetic conjugation approach, the oligonucleotide and peptide components are built on separate supports using standard solid-phase protocols but are conveniently functionalized so that they may be linked together after synthesis. In the stepwise solid-phase approach, the oligonucleotide-peptide conjugate is prepared on a single support using special protecting groups in conjunction with modified synthesis protocols that minimize unwanted side-reactions. In this section, we describe specific protocols that our group has developed in recent years; several alternative protocols reported by others may also be found in the bibliography (5-9). As an example of the scope of the utility of the approaches described in this chapter, **Table 1** lists several oligonucleotide-peptide conjugates that have been produced by our group using these protocols.

Prior to conducting any oligonucleotide-peptide synthesis work, firstly, a decision to employ either a stepwise or a post-synthetic conjugation protocol should be made. Post-synthetic conjugation protocols tend to offer a greater level of flexibility, as peptides can be introduced either at the 3' or 5'-ends of the oligonucleotides or at

internal nucleobases. Moreover, if the peptides are long and complex or if the oligonucleotides are long and highly modified, then post-synthetic conjugation protocols are generally more suitable. Unfortunately, not all types of peptides and oligonucleotides work well in these protocols. For example, these conjugation reactions are not very efficient when using hydrophobic peptides that are not very soluble in aqueous solvents. In addition, low yields have been observed during the conjugation of oligonucleotides to highly structured peptides with several positive charges. On the other hand, stepwise solid-phase protocols are efficient only when using short-length peptides and moderate-sized oligonucleotides. Furthermore, some amino acids such as arginine are difficult to introduce, as there is no good protecting group available for the guanido group that can be removed with base (*see Note 1*). And finally, stepwise protocols are only efficient when synthesizing conjugates with the peptide linked at the 3'-end of the oligonucleotide (*see Note 4*).

Table 1. Example of oligonucleotide-peptide conjugates prepared by using the protocols described in this chapter

Oligonucleotide	Peptide	Protocol	use	Ref.
<i>c-myc</i> ¹	postsynthetic	Non-radioactive labeling, 2D-DNA arrays	13
<i>c-myc</i> ¹	stepwise	Non-radioactive labeling	14, 15
NLS SV40 ² Nucleoplasmine ³	postsynthetic	Antisense inhibition of gene expression	1, 4
Nucleoplasmin ³ -(Lys) _n - n= 2,4,8 -(Arg) _n - n= 2,4,8	stepwise	Antisense and siRNA inhibition of gene expression	3, 4, 12
<i>fos</i> ⁴	postsynthetic	Binding to <i>jun</i>	16

¹Peptide epitope recognized by the monoclonal antibody from clone 9E10; ²Nuclear localization sequence of simian virus SV40; ³Nuclear localization sequence of nucleoplasmin; ⁴Peptide fragment 35 amino acids) containing the leucine-zipper region of *fos*.

3.1. Preparation of Oligonucleotide-Peptide Conjugates by Stepwise Solid-Phase

Synthesis

In the stepwise protocol presented below, oligonucleotide-peptide conjugates were synthesized on a polyethylene glycol-polystyrene (PEG-PS) support. This support was selected since it gave the best results for the coupling of amino acids and nucleoside phosphoramidites. **Figure 3** outlines the general procedure for the

stepwise synthesis of oligonucleotide-peptide conjugates on the same support. To avoid the use of strong acids in the presence of the oligonucleotide, the peptide component was synthesized first using the acid-labile Boc group to protect the α -amino function. The protecting groups for the side chains of the amino acids (Fmoc for Lys and Fm for Asp) and the linker for the first amino acid to the support were base-labile so that they could be removed at the same time as the protective groups for the nucleobases. To this end, 6-aminohexylsuccinyl was utilized as the linker molecule, yielding a peptide with an aminohexylamide group at the C-terminal.

Figure 3. Outline of the preparation of oligonucleotide-3'-peptide conjugates using stepwise solid-phase synthesis. **a)** Boc-NH-(CH₂)₆-OCO-(CH₂)₂-COOH, PyBOP; **b)** Peptide synthesis using Boc-amino acids, PyBOP; **c)** Trityl-O-(CH₂)₃-COOH, PyBOP; **d)** Oligonucleotide synthesis, ammonia deprotection and HPLC purification.

Once the peptide had been synthesized, the spacer molecule 4-*O*-trityl 4-hydroxybutyric acid was incorporated to allow the peptide to connect with the oligonucleotide component. This spacer molecule introduces a hydroxyl group protected by a trityl group at the *N*-terminal position of the peptide. After the addition of the linker, the oligonucleotide sequences were assembled. At the end of the synthesis, the oligonucleotide-peptide conjugates were purified by HPLC and characterized by matrix-assisted laser desorption/ionisation-time of flight (MALDI-TOF) mass spectrometry (MS). Note that this protocol may be used for the synthesis of either DNA or RNA

sequences for the oligonucleotide component; in the latter case, the RNA monomers are protected with a *t*-butyldimethylsilyl (TBDMS) group. Furthermore, oligodeoxynucleotides carrying lysine or ornithine residues can have these converted to homoarginine or arginine by reaction with *O*-methylisourea.

3.1.1. Solid-phase synthesis of the peptide moiety

1. Boc-6-aminohexyl hemisuccinate was synthesized as follows: Boc-aminohexanol (1 eq) was reacted with succinic anhydride (1.5 eq) and DMAP (1.5 eq) in DCM at room temperature overnight to form the corresponding hemisuccinate. The mixture was next dissolved with additional DCM and treated with a solution of 0.1 M NaH₂PO₄ (pH 5). The organic layer was dried with anhydrous sodium sulphate and concentrated to dryness. The resulting compound was used in the next step without further purification.
2. Amino-polyethylene glycol-polystyrene solid (PEG-PS) support was washed with dichloromethane (DCM) (4 x 30 s); trifluoroacetic acid (TFA) / DCM (2:3, v/v; 1 x 1 min; 1 x 30 min); DCM (4 x 30 s); *N,N*-diisopropylethylamine (DIEA) / DCM (1:19, v/v; 3 x 1 min) and DCM (4 x 30 s).
3. Boc-6-aminohexyl hemisuccinate (synthesized in **Step 1**) was reacted with the solid support for 2 h at room temperature using the following mixture: PyBOP (5.0 eq), DIEA (10.0 eq), and hemisuccinate (5.0 eq). This mixture was previously pre-activated (5 min) in *N,N*-dimethylformamide (DMF) and introduced later onto the solid support. After the coupling of the hemisuccinate, the resulting support was acetylated using a mixture of acetic anhydride / DIEA / DMF (1:1.7:15.3, v/v/v; 15 min)
4. The peptide chain was elongated manually in DMF using a 5-fold molar excess of amino acid Boc-protected amino acids and a 5-fold molar excess of PyBOP and a 10-fold molar excess of DIEA for 1 h. The Boc group was removed using the following procedure: Solid-phase support was treated with DCM (4 x 30 s); TFA / DCM (2:3, v/v; 1 x 1 min; 1 x 30 min); DCM (4 x 30 s); DIEA / DCM (1:19, v/v; 3 x 1 min) and DCM (4 x 30 s). Boc-amino acids were protected with base-labile groups.
5. The trityl derivative of 4-hydroxybutyric acid was synthesized as follows (*see* **Note 5**): 1 eq of 4-hydroxybutyric acid was reacted with 1.4 eq of triphenylchloromethane (trityl chloride) in anhydrous pyridine overnight at 50 °C. The reaction was stopped by the addition of methanol, and the solvent was evaporated. Silica gel chromatography using DCM with an increasing amount of methanol (0-5%) plus 1% triethylamine yielded the desired product at 50% yield.
6. Coupling of the trityl derivative of 4-hydroxybutyric acid to the peptide: The addition of this building block introduces a hydroxyl group to the existing peptide. This hydroxyl group is required for the subsequent

assembly of the oligonucleotide (see **Subheading 3.1.2** below). The linker was coupled to the peptide-support using PyBOP and DIEA as described above in **Step 3**. Afterwards, any remaining unreacted amino groups on the support were blocked with acetic anhydride and DIEA.

3.1.2. Synthesis of oligonucleotides carrying peptide sequences at the 3'-ends

1. The peptide-modified supports prepared in **Subheading 3.1.1** above were next employed for the synthesis of the oligonucleotide sequences in a DNA synthesizer using the following procedure: 2-Cyanoethyl phosphoramidites were dissolved (to give 0.1 M solutions) in either dry DCM, or acetonitrile, or with a mixture of ACN / DCM (8:2, v/v) (*see Note 6*). A modified synthesis cycle was used in which the coupling time was increased to 5 min, the capping and oxidation times to 1 min, and the detritylation time to 2 min (4 x 30 s). In the DNA sequences, the last 4,4'-dimethoxytrityl (DMT)-protecting group was not removed. In the RNA sequences, the last DMT group was removed because the DMT is partially lost during the fluoride treatment (*see Note 7*). The average coupling yield was ~ 97-98% per step for RNA monomers and 99% for 2'-*O*-methyl-RNA and DNA monomers.
2. The solid supports containing the oligonucleotide-peptide conjugates were washed with acetonitrile, treated with a solution containing 0.5 M 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in ACN for 5 min, washed with ACN, and then dried (*see Note 8*). In order to deprotect conjugates containing DNA oligonucleotides, **Step 3** below was next carried; for conjugates containing RNA oligonucleotides, **Step 4** was carried out instead.
3. For the deprotection of DNA-peptide conjugates: The solid supports were treated with concentrated aqueous ammonia overnight at 55 °C and then recovered by filtration. After filtration, the supports were washed with water and the combined solutions were evaporated to dryness. Following this, HPLC purification of the DNA-peptide conjugates was carried out by proceeding directly to **Step 5** below.
4. For the deprotection of RNA-peptide conjugates: The solid supports were treated with concentrated aqueous ammonia-ethanol (3:1, v/v) for 1 h at 55 °C and then recovered by filtration. After filtration, the supports were washed with ethanol and the combined solutions were evaporated to dryness. The supports were next treated with 0.15 ml of triethylamine tris(hydrofluoride) / triethylamine / *N*-methylpyrrolidone (4:3:6, v/v/v) at 65 °C to remove the TBDMS groups. After incubating for 2.5 h at 65 °C, the reactions were stopped by the addition of 0.3 ml of isopropoxytrimethylsilane and 0.75 ml of diethyl ether. The resulting mixtures were stirred and cooled down to 4 °C, which led to the formation of a precipitate. The precipitate

material was collected by centrifugation at $4500 \times g$ for 5 min at 4 °C, washed with ether and then centrifuged again. Finally, the collected residues were dissolved in water and the RNA-peptide conjugates were purified by HPLC as described in **Step 5** below.

5. Reversed-phase HPLC purification of oligonucleotide-peptide conjugates was performed using a Nucleosil® 120-10 C₁₈ column (250 x 4 mm) under the following conditions for solvent A and solvent B: 20 min linear gradient from 15% to 80% solvent B, and 5 min 80% solvent B (DMT-on conditions); 20 min linear gradient from 0% to 50% B (DMT-off conditions); flow rate of 3 ml/min.
6. Characterization of oligonucleotide-peptide conjugates by mass spectrometry (MS) was performed as follows: MALDI-TOF MS spectra were acquired with a mass spectrometer equipped with a nitrogen laser at 337 nm using a 3-ns pulse. The matrix contained 2,4,6-trihydroxyacetophenone (THAP; 10 mg/ml in ACN / water, 1:1, v/v) and ammonium citrate (50 mg/ml in water) at a ratio of 9:1 (v/v).
7. Analytical reversed-phase HPLC was performed using a Waters XBridge™ OST C₁₈ column (2.5 μm, 4.6 x 50 mm) column under the following conditions for solvent A and solvent B: 10 min linear gradient from 0% to 35% B; flow rate of 1 ml/min.
8. Characterization of oligonucleotide-peptide conjugates by denaturing polyacrylamide gel electrophoresis was performed as follows: Polyacrylamide gels (8 M urea; 20%, acrylamide / bisacrylamide ratio = 19:1; 14 x 16 x 0.1 cm) were run for 5 h at 400 V. Three micrograms of oligonucleotide-peptide conjugates were loaded in each lane of the gel. Following electrophoresis, gels were stained with Stains-All solution to visualize the oligonucleotide-peptide conjugate bands.

Figure 4. A representative HPLC purification profile for an oligonucleotide-3'-peptide conjugate product obtained by stepwise synthesis

3.1.3 Introduction of guanidine groups

Oligodeoxynucleotide-peptide conjugates carrying lysine or ornithine residues can have these be converted to homoarginine or arginine by reaction with *O*-methylisourea as follows:

1. Oligodeoxynucleotide-peptide conjugates containing unprotected amines (~4.0 OD) were treated with a mixture (125 μ l) prepared by combining 100 μ L of a solution containing 1 g/mL *O*-methylisourea chloride in water with aqueous ammonia (32%, w/w; 125 μ L).
2. After heating the above reaction mixture at 55 °C overnight, the solvent was removed.
3. The resulting modified conjugates were desalted by Sephadex gel filtration, and further purified by preparative HPLC (*see Subheading 3.2, Step 5*).

3.2. Preparation of Oligonucleotide-Peptides Conjugates by Post-Synthetic Conjugation of Peptides with Oligonucleotides Carrying Thiol Groups

1. Firstly, a peptide carrying maleimido groups was synthesized using a Rink-Amide polystyrene support (0.56 mmol/g loading) and Fmoc-amino acids. The Fmoc protecting groups were removed by treating the resin with a 1:1 (v/v) solution of piperidine / DMF for 2 min, and then for 8 min with a solution of 20 % (v/v) piperidine in DMF. Following this, the support was washed four times with DMF. Next, a mixture containing 4 eq of Fmoc-protected amino acid (0.9 mmol), 4 eq of *N*-hydroxybenzotriazole (HOBt) (0.121 g, 0.9 mmol), 4 eq of 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (0.34 g, 0.9 mmol), 8 eq of DIEA (312 ml, 1.8 mmol) and a small amount of DMF was preactivated for 5 min before addition to the resin. The completion of the coupling reaction was qualitatively checked with the ninhydrin test. To block unreacted amino groups, a mixture of acetic anhydride (1ml), DIEA (1.7 ml) and DMF (15.3 ml) was added for 15 min.
2. After completion of the peptide sequence, 6 eq of 3-maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS) and 6 eq of HOBt in a small amount of DMF were added. The maleimido-peptide was deprotected and released from the resin by treatment with TFA containing 5% (v/v) water for 4 h at room temperature. Following this, the resin was filtered and washed several times with water. The combined solutions were evaporated under reduced pressure. The resulting residue was washed three times in diethyl ether and concentrated to dryness to eliminate the excess TFA.
3. Oligonucleotides carrying a thiol group at either the 3'-end, the 5'-end or at an internal nucleobase position were synthesized on a DNA synthesizer using the standard β -cyanoethyl-protected phosphoramidite method

by following protocols recommended by the supplier. The oligonucleotides were removed from the solid support by treatment with concentrated ammonia containing 0.1 M dithiothreitol (DTT) for 6 h at 55°C (*see Step 4* below). Under these conditions, all base-protecting groups were also cleaved.

Figure 5. Outline of the preparation of oligonucleotide-peptide conjugates using the post-synthetic conjugation method. An oligonucleotide carrying a thiol group either at the 3'- or 5'-ends, or at an internal nucleobase is assembled using conventional solid-phase synthesis protocols. The use of a 0.1 M DTT solution during ammonia deprotection removes all the protecting groups and also cleaves the disulfide bond, yielding oligonucleotides with a free thiol group that can react with maleimido-modified peptides.

4. To prevent side reactions, the oligonucleotides and maleimido-modified peptides were cleaved from the solid support just before use. For the same reason, the maleimido-peptide was used without further purification. After synthesis, the oligonucleotide was treated with a solution (1 ml) of 0.1 M DTT in concentrated ammonia at 55 °C overnight. Under these conditions, the *tert*-butyl thiol-protecting group was removed. The solution was next concentrated to dryness. Following this, the residue thus obtained was dissolved in sterile water. DTT was removed from the crude oligonucleotide solution by using a NAPTM-10 desalting column. The oligonucleotide was eluted with 1.5 ml sterile water. The purified oligonucleotide solution was then mixed with 0.5 ml of solvent A containing a 10-fold molar excess of the maleimido-peptide. To adjust the solution to pH = 6.5, a few drops of 1 M aqueous TEAA solution (pH=6.5) were added (*see Note 9*). After 10 h at room temperature, the reaction mixture was concentrated to dryness, dissolved in sterile water and purified with HPLC. The final oligonucleotide-peptide conjugate was characterized by mass spectrometry.

Figure 6. A representative HPLC purification profile for the conjugation products obtained by reacting a *N*-maleimido-peptide (15 amino acids) with a 5'-thiol-oligonucleotide (8 bases). The desired oligonucleotide-peptide conjugate appears as a double peak (two isomers) eluting at approximately 11 min. The peak eluting at ~9 min is the starting thiol-modified oligonucleotide. For conjugates that contain longer oligonucleotides, the separation of the two isomers cannot typically be observed.

4. Notes

1. The protection of arginine is difficult, as there is no good protecting group available for the guanidine group that can be removed in the presence of oligonucleotides. The use of a Di-Fmoc derivative of arginine has been reported by others (*17*); here, we describe a protocol for the guanidinylation of ornithine-peptides that yields arginine-peptides in excellent yields. This protocol can also be used for the synthesis of homoarginine peptides from lysine peptides.
2. There are several companies, such as Applied Biosystems and Sigma-Aldrich, that supply conventional phosphoramidites, ancillary reagents and solid supports for oligonucleotide synthesis. In addition to the standard reagents, more elaborated phosphoramidites and solid supports can be obtained from companies that specialize in oligonucleotide reagents, such as Link technologies (Scotland), Glen Research (Sterling, VI, USA), Berry & Associates (Dexter, MI, USA), TriLink Biotechnologies (San Diego, CA, USA), Nedken (Foster City, CA, USA), ChemGenes (Wilmington, MA, USA), Prime Synthesis (Aston, PA, USA), and Metkinen (Finland).
3. Additionally, it is possible to use a third method, known as solid phase fragment condensation (SPFC), in which a partially protected peptide fragment is coupled directly to an oligonucleotide on a solid support

(10).

4. Protocols for the assembly of peptides at the 5'-end of oligonucleotides have been described previously (18, 19). In general, peptide assembly at the 5'-end of oligonucleotides is less efficient due to side reactions such as acetylation.
5. 6-hydroxyhexanoic acid and 12-hydroxydodecanoic acid derivatives can be prepared in a similar manner.
6. We have observed that the specific peptide sequence present on the solid-phase support can influence coupling yields during oligonucleotide synthesis. Supports carrying a large number of lysines protected with Fmoc groups show less efficient coupling when phosphoramidites are dissolved in acetonitrile. In this case, coupling efficiencies are improved if the phosphoramidites are dissolved in dichloromethane. Depending on the peptide sequence, either ACN, DCM or mixtures of both solvents may be used.
7. The use of tetrabutylammonium fluoride solution instead of triethylamine tris(hydrofluoride) does not remove the last DMT; however, removal of the silyl groups is slower.
8. The DBU treatment eliminates the cyanoethyl groups from phosphates. This step is important to avoid the addition of acrylonitrile to lysine residues.
9. During the conjugation of 5'-thiol oligonucleotides with a peptide carrying the leucine-zipper region of fos, we have observed low yields using triethylammonium acetate buffers. However, using 10 mM Tris.HCl, 100 mM NaCl, pH 7.5 as the buffer improved the yields dramatically.

Acknowledgments

This work is supported by the Spanish Ministry of Education (BFU2007-63287/BMC), Generalitat de Catalunya (2009/SGR/238), Instituto de Salud Carlos III (CIBER-BNN), and European Communities (DYNAMO contract 028669 (NEST), FUNMOL NMP4-SL-2009-213382).

References

1. Eritja, R., Pons, A., Escarceller, M., Giralt, E., and Albericio, F. (1991) Synthesis of defined peptide-oligonucleotide hybrids containing a nuclear transport signal sequence. *Tetrahedron* **47**, 4113-4120.
2. Soukchareun, S., Tregear, G.W., and Haralambidis, J. (1995) Preparation and characterization of antisense oligonucleotide-peptide hybrids containing viral fusion peptides. *Bioconjug Chem* **6**, 43-53.
3. de la Torre, B.G., Aviñó, A., Tarrasón, G., Piulats, J., Albericio, F., and Eritja, R. (1994) Stepwise solid-phase synthesis of oligonucleotide-peptide hybrids. *Tetrahedron Lett* **35**, 2733-2736.

4. de la Torre, B.G., Albericio, F., Saison-Behmoaras, E., Bachi, A., Eritja, R. (1999) Synthesis and binding properties of oligonucleotides carrying nuclear localization sequences. *Bioconjug Chem* **10**, 1005-1012.
5. Tung, C.H., and Stein, S. (2000) Preparation and applications of peptide-oligonucleotide conjugates. *Bioconjug Chem* **11**, 605-618.
6. Eritja, R. (2000) Synthesis of oligonucleotide-peptide conjugates and nucleopeptides. *Solid-Phase Synthesis. A practical guide.* (S. Kates, F. Albericio Eds) Marcel Dekker, New York, pp529-548.
7. Grandas, A., Marchán, V., Debéthune, L., and Pedroso, E. (2004) Stepwise solid-phase synthesis of nucleopeptides. *Current Protocols in Nucleic Acid Chemistry*, John Willey & Sons, NY, Chapt. 4.22.1–4.22.54.
8. Röglin, L., Seitz, O. (2008) Controlling the activity of peptides and proteins with smart nucleic acid-protein hybrids. *Org Biomol Chem* **6**, 3881-3887.
9. Venkatesan, N., and Kim, B.H. (2006) Peptide conjugates of oligonucleotides: synthesis and applications. *Chem Rev.* **106**, 3712-3761.
10. Gait, M.J. (2003) Peptide-mediated cellular delivery of antisense oligonucleotides and their analogues. *Cell Mol Life Sci* **60**, 844-853.
11. Brumcot, D., Manoharan, M., Koteliensky, V., and Sah, D.W. (2006) RNAi therapeutics: a potential new class of pharmaceutical drugs. *Nat Chem Biol* **2**, 711-719.
12. Aviñó, A., Ocampo, S. M., Caminal, C., Perales, J.C., and Eritja, R. (2009) Stepwise synthesis of RNA conjugates carrying peptide sequences for RNA interference studies. *Mol Divers* **13**, 287-293.
13. Gottschling, D., Seliger, H., Tarrasón, G., Piulats, J., Eritja, R (1998) Synthesis of oligodeoxynucleotides containing N⁴-mercaptoethylcytosine and their use in the preparation of oligonucleotide-peptide conjugates carrying c-myc tag sequence. *Bioconjug Chem* **9**, 831-837.
14. Frieden, M., Aviñó, A., Tarrasón, G., Escorihuela, M., Piulats, J., Eritja, R. (2004) Synthesis of oligonucleotide-peptide conjugates carrying the c-myc peptide epitope as recognition system. *Chem Biodivers* **1**, 930-938.
15. Merkoçi, A., Aldavert, M., Tarrasón, G., Eritja, R., Alegret, S. (2005) Toward an ICPMS-Linked DNA assay based on gold nanoparticles immunoconnected through peptide sequences. *Anal Chem* **77**, 6500-6503.
16. Portela, C., Albericio, F., Eritja, R., Castedo, L., Mascareñas, J.L. (2007) Ds-Oligonucleotide-peptide conjugates featuring peptides from the leucine zipper region of fos as switchable receptors for the

oncoprotein Jun. *ChemBioChem* **8**, 1110-1114.

17. Debethune, L., Marchan, V., Fabregas, G., Pedroso, E. Grandas, A. (2002) Towards nucleopeptides containing any trifunctional amino acid (II) *Tetrahedron* **58**, 6955-6978.
18. Zaramella, S.; Yeheskiely, E.; Strömberg, R. (2004) A method for solid-phase synthesis of oligonucleotide 5'-peptide-conjugates using acid-labile alpha-amino protections. *J Am Chem Soc* **126**, 14029-14035.
19. Ocampo, S. M., Albericio, F., Fernández, I., Vilaseca, M., Eritja, R. (2005) A straightforward synthesis of 5'-peptide oligonucleotide conjugates using N^a-Fmoc-protected amino acids. *Org Lett* **7**, 4349-4352.