Template-directed ligation of peptides to oligonucleotides

Richard K Bruick¹, Philip E Dawson¹, Stephen BH Kent¹, Nassim Usman² and Gerald F Joyce¹*

¹The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037, USA and ²Ribozyme Pharmaceuticals Incorporated, 2950 Wilderness Place, Boulder, CO 80301, USA

Background: Oligonucleotide–peptide conjugates have several applications, including their potential use as therapeutic agents. We developed a strategy for the chemical ligation of unprotected peptides to oligonucleotides in aqueous solution. The two compounds are joined via a stable amide bond in a template-directed reaction.

Results: Peptides, ending in a carboxy-terminal thioester, were converted to thioester-linked oligonucleotide–peptide intermediates. The oligonucleotide portion of the intermediate binds to a complementary oligonucleotide template, placing the peptide in close proximity to an adjacent template-bound oligonucleotide that terminates in a 3' amine. The ensuing reaction results in the efficient formation of an amide-linked oligonucleotide–peptide conjugate.

Conclusions: An oligonucleotide template can be used to direct the ligation of peptides to oligonucleotides via a highly stable amide linkage. The ligation reaction is sequence-specific, allowing the simultaneous ligation of multiple oligonucleotide–peptide pairs.

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Introduction

Synthetic oligonucleotides and peptides have enjoyed a wide range of applications in both biology and chemistry. As a consequence, oligonucleotide–peptide conjugates have received considerable attention, most notably in the development of antisense constructs with improved pharmacological properties [1–7]. In addition, oligonucleotide–peptide conjugates have been used as molecular tags [8–11], in the assembly of supramolecular arrays [12] and in the construction of encoded combinatorial libraries [13–15]. To make these chimeric molecules more accessible for a broad range of investigations, we sought to develop a facile method for joining fully deprotected oligonucleotides and peptides through a stable amide bond linkage. Furthermore, we wished to make this ligation reaction addressable, enabling one to direct the ligation of specific oligonucleotide and peptide components. To confer specificity and accelerate the rate of the reaction, the ligation process was designed to be dependent on the presence of a complementary oligonucleotide template.

The ligation chemistry used here is related to a method developed previously for the native chemical ligation of unprotected peptides in solution ([16]; see also [17] for commentary). The method of native chemical ligation for joining two unprotected polypeptides involves a chemoselective reaction between one polypeptide bearing a thioester at the carboxyl terminus and a second polypeptide bearing a cysteine residue at the amino terminus. Thioester exchange gives rise to a thioester–linked intermediate, which undergoes a spontaneous rearrangement, under the same reaction conditions, to form a stable peptide bond at the ligation junction. This reaction is facilitated by the favorable orientation of the α-amine of the cysteine residue relative to the thioester linkage; these functionalities are arranged in an intramolecular five-membered ring.

Reactions between two oligonucleotides in solution can be accelerated when carried out in a template-directed fashion. A nucleic acid template is used to hold the oligonucleotides in close proximity while a condensing agent is added to promote the joining of their adjacent ends. The template confers sequence-specificity through base-pairing interactions and greatly enhances the efficiency of condensation by increasing the effective concentration of the bound substrates. Template-directed chemical ligation has been used to join oligonucleotides by either a phosphodiester or a non-native linkage [18–20]. It would be desirable to apply the advantages of template-directed condensation to chemical ligation reactions involving non–oligonucleotide compounds. In the present study, we used a DNA template to direct the ligation of a peptide to an oligonucleotide, the peptide being presented by a second oligonucleotide in the form of a reactive thioester-linked intermediate.

The reaction scheme is illustrated in Figure 1. As in native chemical ligation of polypeptides, there is an initial chemoselective reaction between a thiol and an unprotected peptide that contains an activated carboxy-terminal thioester. In this case, however, the thiol lies at the 5' terminus of a 5'-deoxy,5'-thio oligonucleotide (DNA1). This transthioesterification reaction results in formation of an oligonucleotide–peptide intermediate, joined via a

*Corresponding author.
Fig. 1. Scheme for template-directed ligation of a peptide to an oligonucleotide. (a) The peptide, ending in a carboxy-terminal thioacid, is converted to the activated thioester upon incubation with Ellman’s reagent (RS-SR). This in turn reacts with a 5'-thiol-terminated oligonucleotide (DNA1), resulting in formation of a thioester-linked intermediate. The DNA1–peptide intermediate is held in close proximity to a 3'-thiol-terminated oligonucleotide (DNA2) by hybridization to adjacent sites on a complementary template (TEM). (b) The nucleophilic amine attacks the carbonyl carbon, forming an amide bond between DNA2 and the peptide, and releasing the 5'-thiol-terminated DNA1.

The template compensates for the lack of a highly activated carbonyl by holding the 3' amine in close proximity to the 5' thioester, fulfilling a function analogous to that of the 5-membered cyclic intermediate in native chemical ligation of polypeptides. The thiol-containing oligonucleotide (DNA1) is restored as the amide-linked oligonucleotide–peptide product is formed. DNA1 then has the potential to form another thioester-linked intermediate; both it and the template may be recycled following the reaction. The amide-linked oligonucleotide–peptide product can be separated from the other components by standard purification techniques, such as polyacrylamide gel electrophoresis. The ligation reaction is efficient and quite general, as demonstrated in this study by the use of two different peptides and two different combinations of complementary oligonucleotides.

Results

A list of oligonucleotides and peptides that were used in this study is provided in Table 1. The 3'-amine-terminated DNA2 was prepared by enzymatic addition of 3'-amino-2',3'-dideoxythymidylate to the 3' terminus of d(GGCCCTCoH) using terminal transferase. The 5'-thiol-terminated DNAs 1A and 1B were synthesized by standard phosphoramidite chemistry, incorporating S-trityl uridine at the 5' terminus. The 5'-trityl protecting group was thioester linkage. Next, the thioester-linked intermediate is brought into close proximity to a 3'-amine-terminated oligonucleotide (DNA2) by annealing to a DNA template (TEM) that is complementary to both oligonucleotides. A chemical ligation reaction ensues, with the 3' amine of DNA2 attacking the carbonyl carbon of the DNA1–peptide intermediate, resulting in formation of an amide bond.

Table 1. Oligonucleotides and peptides used in this study.

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Name</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>DNA1A</td>
<td>5'-HS-UATATCGGACTGCC-3'</td>
<td></td>
</tr>
<tr>
<td>DNA1B</td>
<td>5'-HS-UGCGATACTGACAG-3'</td>
<td></td>
</tr>
<tr>
<td>DNA1C</td>
<td>5'-HO-CATCFrCCCCGACCG-3'</td>
<td></td>
</tr>
<tr>
<td>DNA2</td>
<td>5'-GGCCCTCT-NH2-3'</td>
<td></td>
</tr>
<tr>
<td>TEM A</td>
<td>5'-GTGACCTCGCAGTATATAAGGCG-3'</td>
<td></td>
</tr>
<tr>
<td>TEM B</td>
<td>5'-CTGTCAGTATCGCAAGGGCCA-3'</td>
<td></td>
</tr>
<tr>
<td>Peptides</td>
<td>Name</td>
<td>Sequence</td>
</tr>
<tr>
<td>NLS</td>
<td>H$_2$N- Ala Ala Lys Arg Val Lys Leu Gly -COSR</td>
<td></td>
</tr>
<tr>
<td>dyn</td>
<td>H$_2$N- Tyr Gly Gly Phe Leu Arg Arg Gly -COSR</td>
<td></td>
</tr>
</tbody>
</table>

DNA1A and 1B are composed of standard deoxyribonucleotides except for a single 5'-deoxy,5'-thio,2'-methoxy-uridine residue at the 5' terminus. DNA2 is composed of standard deoxyribonucleotides except for a single 3'-amino, 2',3'-dideoxythymidine residue at the 3' terminus. The peptides were prepared with a carboxy-terminal glycine thioacid and subsequently converted to the 5-thio-2-nitrobenzoic acid ester (-COSR) upon treatment with Ellman’s reagent.
Fig. 2. Formation of the thioester-linked oligonucleotide-peptide intermediate. DNA 1A, 1B, and 1C were radiolabeled at the 3′ terminus with [γ-32P]dATP and terminal transferase. The 5′-trityl protecting group was removed from DNA 1A and 1B as described in Materials and methods. DNA 1C contains a 5′-hydroxyl rather than 5′-thiol. DNA 1A was added to a reaction mixture containing ~850 μM peptide, 30 mM sodium phosphate (pH 8.0), and 2.3 mM spermidine, which was incubated at 25 °C for 30 min. Reaction products were analyzed by electrophoresis in a 17.5% polyacrylamide/8 M urea gel with a running buffer containing 40 mM 4-morpholinepropane sulfonic acid (pH 6.5), 45 mM borate, and 0.5 mM EDTA. The products were visualized by autoradiography. N, NLS peptide; D, dyn peptide.

removed before using the oligonucleotide. The peptides were prepared by stepwise solid-phase synthesis on a thioester resin. The carboxy-terminal thioacid of the deprotected peptide was converted to the 5-thio-2-nitrobenzoic acid ester upon incubation with Ellman's reagent (5,5'-dithio-bis(2-nitrobenzoic acid)). The peptides were purified and characterized by HPLC and electrospray mass spectrometry prior to use.

Thioester formation
The first step of the reaction involves formation of a thioester-linked intermediate between the peptide and the thiol-containing DNA 1. DNA 1A and 1B, radio-labeled at the 3′ terminus, were separately allowed to react with either the NLS or dyn peptide in the presence of 2.3 mM spermidine at pH 8.0 and 25 °C (Fig. 2). The reaction proceeded to 60–70% completion in less than 30 min. A control oligonucleotide that contained a 5′-hydroxyl rather than a 5′-thiol (DNA 1C) did not form the corresponding carbamoylpeptide. A small amount of disulfide-linked DNA 1 dimer was observed, migrating somewhat slower than the oligonucleotide–peptide thioester. The thioester intermediate was stable at room temperature over 24 h, but was degraded significantly at 37 °C over the same time period. The identity of the thioester intermediate was confirmed by electrospray mass spectrometry. For example, the thioester-linked product between DNA 1B and the dyn peptide had an observed molecular weight of 5228.0 ± 0.3 Da, consistent with the expected molecular weight of 5227.8 Da.

Amide-bond formation
After formation of the thioester-linked intermediate, the appropriate template oligonucleotide (TEM) and DNA 2 were added to the reaction mixture. The template was expected to bind both oligonucleotides, positioning the 3′ amine of DNA 2 in close proximity to the 5′ thioester of the DNA 1–peptide intermediate. The ensuing reaction led to the formation of the amide-linked DNA 2–peptide product upon incubation at 37 °C for 24 h (Fig. 3). The reaction proceeded efficiently, producing a single major product in good yield (lanes 3–6). Product formation does not occur in the absence of the complementary template (lane 7) or in the presence of a mismatched template (data not shown). The optimal

Fig. 3. Formation of the amide-linked oligonucleotide-peptide product. [5′-32P]-labeled DNA 2 was incubated with either the free activated peptide (−) or the peptide conjugated to DNA 1A or DNA 1B via a thioester, in either the presence (+) or absence (−) of the corresponding template oligonucleotide. Reaction products were separated by electrophoresis in a denaturing polyacrylamide gel run at pH 8.3, an autoradiogram of which is shown. N, NLS peptide; D, dyn peptide.
reaction conditions (2–4 mM spermidine, pH 8.0, 37 °C) were determined experimentally. A time-course analysis showed that the reaction with the NLS peptide proceeded to 70–85% completion in 15 h (Fig. 4). The reaction with the dyn peptide was less efficient, proceeding to 60–70% completion in 30 h. The DNA 1A–TEMA templating system resulted in slightly greater yields compared to the DNA 1B–TEM B system.

The products of amide bond formation between DNA 2 and the dyn peptide were characterized by matrix-assisted laser desorption ionization (MALDI) mass spectrometry. The completed reaction mixture was purified by reverse-phase column chromatography to remove unreacted peptide and salts. MALDI mass spectrometry was carried out in the negative-ion mode to facilitate detection based on the nucleic acid component of the various compounds. As shown in Table 2, the desired amide-linked oligonucleotide–peptide conjugate was formed. In addition, all of the expected intermediates and products were observed.

To confirm that the 3'-amine of DNA 2 is the reactive nucleophile, the 3'-amine-terminated DNA 2 was replaced by an all-deoxy DNA 2, terminating in a 3'-hydroxyl. Incubation of the DNA 1A–NLS thioester intermediate with TEM A and the all-deoxy DNA 2 resulted in formation of a putative carboxyester-linked DNA 2–NLS conjugate, in slightly lower yield compared to the analogous amide-linked product. The presumed carboxyester-linked product underwent complete hydrolysis upon incubation in the presence of 0.01 M NaOH at 25 °C for 1 h, while the corresponding amide-linked product was unaffected (data not shown). As expected, no product was obtained when the 3'-amine-terminated DNA 2 was substituted by DNA 2 ending in 2'-3'-dideoxythymidine.

The ligation reaction was designed so that formation of the amide-linked product only occurs when the amine-terminated oligonucleotide (DNA 2) is brought into close proximity to a peptide presented in the context of a thioester-linked oligonucleotide–peptide intermediate. This localization is accomplished through sequence-specific, Watson–Crick pairing between a template oligonucleotide and the complementary DNA 1 and DNA 2 oligonucleotides. In principle, this template dependence should allow the simultaneous ligation of various amine-terminated oligonucleotides to specific peptides, each in a sequence-dependent fashion. Figure 5 demonstrates the possibility of simultaneous ligation for the case of two oligonucleotide–peptide combinations. The thioester-linked DNA 1A–dyn and DNA 1B–NLS intermediates were formed and purified by polyacrylamide gel electrophoresis. These two intermediates were incubated together in the presence of DNA 2 and either one or both of the TEM oligonucleotides. In the presence of a single oligonucleotide template (TEM A or TEM B), a single amide-linked product was formed (DNA 2–dyn or DNA 2–NLS, respectively), whereas in the presence of both templates, both amide-linked products were obtained.

Discussion

The use of synthetic oligonucleotides as either antisense or ribozyme therapeutics may be limited by their poor ability to penetrate the cell membrane and become localized within the appropriate cellular compartment. Peptide-mediated targeting of oligonucleotides has been proposed as a possible solution to this problem. Oligonucleotides that are conjugated to particular peptides (or polyamino acids) have been shown to have an enhanced ability to penetrate cells [1–3], resist degradation by intracellular nucleases [5] and augment the hybridization of the oligonucleotide to its target [4,6]. Peptides could also be used to direct oligonucleotides

| Table 2. MALDI mass spectroscopy characterization of the products of template-directed amide bond formation. |
|-------------------------------------------------|---------------------------------|
| Expected mass (Da)                             | Observed mass (Da)              |
| dyn peptide (hydrolysis product)              | 925                             | 924 |
| DNA 2                                           | 2362                            | 2359 |
| DNA 2 + dyn (amide-linked)                     | 3270                            | 3269 |
| DNA 1A                                          | 4311                            | 4312 |
| DNA 1A + dyn (thioester-linked)                | 5219                            | 5220 |
| DNA 1A + DNA 1A (disulfide-linked)             | 8620                            | 8652 |
| TEM A                                           | 9241                            | 9249 |

Ligation of DNA 2 and dyn, using DNA 1A and TEM A. Observed masses were assigned on the basis of the expected molecular weights for the various reaction components and products.
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Fig. 5. Formation of multiple amide-linked DNA2–peptide conjugates in an addressable format. DNA1A–dyn and DNA1B–NLS thioester-linked intermediates were preformed and gel purified. Amide bond formation was carried out in the presence of both preformed intermediates and either one or both TEM oligonucleotides at 32°C for 18 h. Reaction products were separated by electrophoresis in a 20% polyacrylamide/8 M urea gel run at pH 8.3, an autoradiogram of which is shown. The imperfect fidelity of the reaction is likely due to 5'-thiol terminated DNA1, liberated from the thioester-linked intermediate, which subsequently attacks a different DNA1–peptide intermediate, independent of sequence.

to particular intracellular sites [7,21,22]. In one study, for example, an antisense oligonucleotide–peptide conjugate was constructed, joined through a thioester linkage [7]. The oligonucleotide was complementary to a portion of HIV-1 mRNA; the peptide contained a sequence (Lys-Asp-Glu-Leu) known to direct proteins to the endoplasmic reticulum, in principle allowing greater access to mRNA targets in the cytoplasm. In HIV-1-infected cells, the antisense oligonucleotide linked to the peptide exhibited greater antiviral activity compared to the oligonucleotide alone. Conversely, peptides have been used as nucleic acid cleavage agents, directed to a particular RNA or DNA target by an attached complementary oligonucleotide [23,24].

Several schemes have been devised for attaching peptides to oligonucleotides using a synthetic linkage, such as a disulfide [10,21,25] or thioether [9,21–23,26], or by Schiff base formation between the ε-amino group of lysine and an oligonucleotide 2′(3′)-dialdehyde [1]. In addition, there are methods for the solid phase synthesis of peptide–oligonucleotide conjugates [27–31]. We have devised a strategy for joining fully deprotected oligonucleotides and peptides in aqueous solution through a stable amide linkage (Fig. 1). This method relies on an oligonucleotide template to promote the ligation reaction and confer specificity for a particular combination of oligonucleotide and peptide. As a test case, we used two different peptides: dynorphin A 1–7 (dyn), a high-affinity ligand for the β-endorphin receptor [32]; and the nuclear localization signal (NLS), a peptide sequence that causes cytosolic proteins to become imported to the nucleus [33]. When conjugated to an oligonucleotide, these and other signal peptides [34] can be used to target a therapeutic antisense or catalytic nucleic acid to a particular cell type or intracellular compartment.

The template-directed ligation reaction proceeds optimally at pH 8.0 and 37°C. We were concerned that the thioester intermediate might be too unstable under these conditions, but this proved not to be the case. The benefit of working at slightly alkaline pH is that it facilitates deprotonation of the oligonucleotide 3'-amine (pKₐ = 8.2) [35], which is necessary for its attack on the adjacent peptidyl thioester. No further improvement in yield was obtained by increasing the pH from 8.0 to 8.5 or by raising the temperature from 37 to 42°C. A similar reaction scheme can be used to prepare oligonucleotide–peptide conjugates joined with a carboxyester linkage. The carboxyester-linked conjugate is far more labile to alkaline hydrolysis relative to the amide-linked compound, however.

The addition of spermidine resulted in increased yields, although no improvement was obtained by adding Mg²⁺. Spermidine and Mg²⁺ both stabilize DNA duplex structure, but the fact that only spermidine led to increased yields suggests that it may have a more subtle effect on the conformation of the template-bound substrates. The initial oligonucleotide concentrations were chosen so that, at 25°C and in the absence of spermidine, nearly all DNA 2 would be bound to a template that itself was saturated with the DNA1–peptide intermediate. Complex formation was confirmed by gel-shift analysis in a non-denaturing polyacrylamide gel (data not shown). Surprisingly, after the reaction conditions were optimized, a similar gel-shift analysis conducted at 37°C in the presence of spermidine revealed that, at equilibrium, almost none of the amine-terminated oligonucleotide is bound to the template. Since amide bond formation is absolutely dependent on the presence of a complementary template, this suggests that ligation proceeds efficiently during the short time that the two oligonucleotide substrates are co-localized on the template.

In the native chemical ligation of polypeptides [16], a covalent thioester intermediate positions the nucleophilic amine for amide bond formation, proceeding through a favorable intramolecular five-membered ring. In our reaction system, the use of an oligonucleotide template similarly provides 'entropic activation' [36], in effect converting an intermolecular reaction to a
pseudo-intramolecular one. The template and 5'-thiol-terminated oligonucleotides are regenerated during the reaction and, in principle, could be recycled for repeated condensation reactions. Furthermore, while the components of the reaction were designed to result in attachment of the peptide to the 3' terminus of the oligonucleotide, it is likely that they could be rearranged to allow attachment of the peptide to the 5' terminus of the oligonucleotide through the template-directed reaction of an oligonucleotide 3'-peptidyl thioester intermediate and an oligonucleotide 5'-amine.

We have carried out the template-directed ligation of peptides to oligonucleotides with a variety of different peptides, up to eight amino acids in length. For certain small peptides that tend to adopt a cyclic conformation, the amine terminus of the peptide may compete with the amine-terminated oligonucleotide, resulting in cyclization of the peptide rather than ligation of the peptide to the oligonucleotide. The maximum size of a peptide that can be ligated efficiently is not known. The only known amine-terminated oligonucleotide, resulting in cyclization of small peptides that tend to adopt a cyclic conformation, was used in the reactions.

In addition to accelerating the reaction, the oligonucleotide template system allows the reaction to occur in an addressable format. The identity of the peptide is specified by the sequence of the oligonucleotide (DNA1) to which it is linked in the thioester intermediate. The template recognizes a particular DNA 1-specific peptide and DNA2, resulting in their ligation. This template dependence allows one to carry out simultaneous ligation of amine-terminated oligonucleotides and preformed oligonucleotide-peptide thioesters, each in a sequence-dependent fashion. In a combinatorial reaction containing multiple preformed thioester intermediates, the sequence of the template would encode the peptide that is to be ligated, analogous to the way in which an mRNA codon encodes an amino acid during protein synthesis.

**Significance**

The chemistry used in this study allows one to ligate fully-deprotected oligonucleotides and peptides in a sequence-dependent manner. The two components may be joined via a stable amide or more labile carboxyester linkage. Because ligation is carried out in an addressable format, one can simultaneously join various combinations of oligonucleotides and peptides. To demonstrate this, we ligated two different peptides to an oligonucleotide using two different oligonucleotide templates. The peptides used are known to be important in vivo, directing proteins to a cell-surface receptor or to the nucleus. Such peptide–oligonucleotide conjugates might therefore be used to target antisense molecules or nucleic acid catalysts to particular cell types or subcellular compartments. In addition, these oligonucleotide–peptide conjugates may be useful as biochemical tools in the construction of molecular tags in the assembly of supramolecular arrays, and in the preparation of novel substrates for the study of nucleic acid catalysis.

**Materials and methods**

**Oligonucleotides and oligonucleotide analogs**

Synthetic oligonucleotides were purchased from Operon Technologies. DNA 1A and 1B were synthesized by standard phosphoramidite chemistry, the 5'-thiol being introduced by incorporation of S-trityl-2'-O-methoxyuridine, 5'-SP(3'-triphosphorylimethylene)mercapto-5'-deoxy-2'-O-methyluridine-3'-O-(2-cyanoethyl-N,N-disopropylphosphoramidite) was prepared from commercially available 2'-O-methyluridine (J. Matulic-Adamic et al., manuscript in preparation). The oligonucleotides were deprotected, except at the 5'-thiol position, by standard methods, and were purified by polyacrylamide gel electrophoresis and subsequent reverse-phase chromatography. Just prior to use, DNA 1A and 1B were suspended in 0.1 M triethylamine acetate (TEAA), pH 6.5, and 0.15 volumes of 1 M aqueous AgNO3 was added to promote removal of the 5'-trityl protecting group. Following incubation at 25 °C for 30 min, 0.2 volumes of 1 M dithiothreitol (DTT) was added and the mixture was incubated at 25 °C for an additional 5 min. The Ag⁺ salt of DTT was removed by centrifugation and washed twice with 0.1 M TEAA, pH 6.5. The supernatants were combined and excess salt and DTT were removed by reverse-phase chromatography on DuPont Nenisorb 20. The deprotected oligonucleotides were lyophilized and resuspended in H₂O.

DNA 2 was prepared by enzymatic addition of 3'-amino, 3'-deoxythymidine to the 3' terminus of d(GGCCCTC) using terminal transferase and 2',3'-dideoxy, 3'-amino TTP [35].

**Synthesis of thioester peptides**

All peptides were synthesized manually using optimized solid-phase methods [37] on a thioester resin, prepared by a generalized version of the Blake–Yamashiro procedure [38–40]. The resulting deprotected thioester was purified and reacted with 1.2 equivalents of Ellman’s Reagent (5,5'-dithiobis(2-nitrobenzoic acid)) at pH 6.5 for 1 h. The peptides were purified by reverse-phase HPLC (linear gradient of 9 to 45% CH₃CN in 0.1% trifluoroacetic acid (TFA) and H₂O over 40 min). Peptide composition and purity were confirmed by electrospray mass spectrometry. Peptides were resuspended in 30 mM sodium acetate, pH 5.2, at a final concentration of ~20 µg µL⁻¹ and stored at ~20 °C.

**Template-directed formation of amide-linked oligonucleotide-peptide conjugate**

The thioester-linked DNA 1-peptide intermediate was prepared by adding detritylated DNA 1 (135 µM) to a reaction mixture containing ~700 µM peptide, 42 mM sodium phosphate, pH 8.0, and 2–4 mM spermidine, which was incubated at 25 °C for 30 min. The appropriate TEM oligonucleotide was added, and the mixture was incubated at 25 °C for 30 min. The reaction was terminated by addition of dithiothreitol (DTT) and the mixture was loaded onto a TSKgel ODS-250 column.
and [5-32P]-labeled DNA 2 were added to the completed reaction mixture to final concentrations of 33 μM and 3.2 μM, respectively. The reaction mixture was incubated at 37 °C for various times, withdrawing aliquots that were quenched by incubation at 65 °C for 1 min, then stored at −20 °C. Reaction products were separated by electrophoresis in a 20% polyacrylamide/8 M urea gel and quantified by phosphorimager analysis.

Mass spectrometry analysis

The thioester-linked conjugate between DNA 1B and the dyn peptide was purified by reverse-phase chromatography on DuPont Nensorb 20 and resuspended in 45% acetonitrile and 0.1% TFA to an approximate concentration of 25 μM. Spectra were obtained using a Sciex API-III quadrupole ion-spray mass spectrometer in the negative ion mode. The amide-linked products were analyzed by MALDI mass spectrometry. Following formation of the amide-linked product, the entire reaction mixture was purified by reverse-phase chromatography and resuspended in a saturated matrix solution of 3-hydroxypicolinic acid (prepared in a 1:1 mixture of acetonitrile and 0.1% TFA in H2O) such that the concentration of the amide-linked conjugate was ~1.5 μM. MALDI samples were prepared by adding 2 μl of this mixture on a stainless steel probe tip (3.1 mm2) before allowing the solvent to evaporate at room temperature. Desorption and ionization of samples were performed with a Lumonics Model HY 400 Nd:YAG laser and spectra were recorded using a Vectec Model VT 2000 laser desorption, linear time-of-flight mass spectrometer. All MALDI spectra were obtained in the negative-ion mode and summed over 50 laser shots. The singly- and doubly-charged molecular spectra were obtained in the negative-ion mode and summed by adding 2 I_1 of this mixture on a stainless steel probe tip.

References


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