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A Catalytic Approach for the Synthesis of Peptide–Oligonucleotides Conjugates in Aqueous Solution or On-Column

Marion Gras,^[a] Pauline Adler,^{*,[a]} and Michael Smietana^{*,[a]}

Peptide–oligonucleotide conjugates (POCs) are covalent architectures composed of a DNA or RNA molecules linked to a peptide. These constructs have found widespread applications ranging from hybrid nanomaterials to gene-targeted therapies. Considering the important role of POCs, a new catalytic approach for their preparation is reported here, that could be applied either on solid support in anhydrous media, or post-synthetically in aqueous buffer. Single amino acids, peptides and cell penetrating peptides (CPPs) were conjugated to various

oligo(ribo)nucleotides with high conversions and good isolated yields. The applicability of the method was demonstrated on more than 35 examples including an analogue of a commercial therapeutic oligonucleotide. Other conjugation partners, such as deoxycholic acid and biotin were also successfully conjugated to oligonucleotides. To highlight the potential of this catalytic approach, these conditions have been applied to iterative processes, which is of high interest for the development of DNA-Encoded Libraries.

Introduction

Synthetic oligonucleotides (ONs) are now part of the therapeutic arsenal for treating various diseases, often associated with undruggable proteins.^[1,2] Composed of around 20 to 50 deoxy- or ribonucleotides, therapeutic oligonucleotides could be single- or double-stranded. However, they suffer from some shortcomings that prevent them from fulfilling their full therapeutic potential,^[3] such as cellular uptake due to the numerous negative charges, stability towards nucleases in the biological fluids or intracellular trafficking.^[4] The development of effective and non-toxic vectors for ON-based drugs has thus emerged as a major concern in both academic and industrial research settings. Many biomolecules have been proposed for improving metabolic stability and delivery.^[5,6] Amongst them, peptide–oligonucleotide conjugates (POCs) (Figure 1a) have proven their superior potential in various ON-based strategies,^[7–9] such as antisense ONs,^[10,11] miRNAs,^[12] siRNAs,^[13] or aptamers.^[14] The use of POCs in self-assembling systems and hybrid nanomaterials, is also growing rapidly with DNA acting as a programmable scaffold capable to positioning peptides with nanoscale precision.^[15–17]

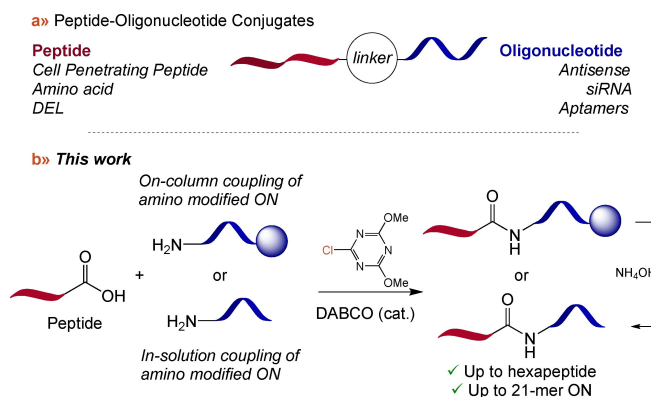


Figure 1. Peptide–Oligonucleotide Conjugates.

Considering the importance of POCs, it is therefore not surprising that numerous access routes have been developed to conjugate a peptide core to an oligonucleotide.^[1,18,19] Among them two main strategies dominate: i) the stepwise solid-phase synthesis of both peptide and oligonucleotide mostly on controlled pore glass (CPG) beads,^[20,21] more rarely on resins, such as polyethylene glycol–polystyrene or Tentagel resins,^[22,23] ii) the post-synthetic conjugation of assembled peptide and oligonucleotide fragments, commonly in solution or more rarely on solid-support.^[22] The advantages of the first strategy are the convenient manipulations and easier purification operations.^[24] However, protecting group incompatibilities often hampers the efficiency of these approaches. Indeed, in most cases, the Fmoc/t-Bu strategy is used for the peptides' synthesis.^[22] The second strategy generally performed in solution avoids chemical incompatibilities but requires the installation of a linker, to assure the unique and selective conjugation of the peptide to the ON. Lots of efforts were made to introduce a large variety of linkers between the peptide and ON fragments, including selenoesters,^[25] thioethers, disulfides,^[26–28] oximes, thiazolidine

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or hydrazones from aldehyde groups,^[29–31] or cyclic cores, obtained by Click-reaction,^[17,32,33] or Diels-Alder adducts.^[34] In the context of small organic molecules chemistry, sustainable methods for the formation of amides are particularly required considering the high environmental impact of classical methods involving low atom economy activating agents such as HATU or EDCI. Greener approaches involving mechanochemistry,^[35–37] neat reactions^[38] or catalysis^[39–43] have also been recently developed. The nature of the solvents used is also an important parameter and amide synthesis in water represents an alternative to highly toxic and hazardous solvents that are commonly used.^[44] “Fast amide couplings in water” has been successfully developed using either classical activating agent (EDC, COMU for instance) in an aqueous micellar media,^[45–47] on resin microwave-promoted reactions,^[48] or using new water-compatible reagents^[49] such as *N*-carboxyanhydrides,^[50] Oxyma derivatives,^[51] or DMTMM (4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride). Despite all these advances, the introduction of an amide bond between an oligonucleotide and a peptide by conventional activating agents such as EDC, HATU or DMTMM remains the most widely used and most extensively studied approach.^[52,53] In fact, their use has even increased since the development of DNA-Encoded Library (DEL) technology.^[54]

Described for the first time in 1992 by Brenner and Lerner for the synthesis and screening of large collections (10⁵ to 10⁸ members) of small molecule compounds linked to a DNA fragment acting as a tag, they showcased the method by preparing tripeptides in an iterative way.^[55,56] Since then, Liu and coworkers extended the application of the technology to macrocyclic peptides,^[57,58] those continuing to be intensively studied.^[59–61] The most common starting material for DEL's synthesis is an amine-terminated headpiece ON.^[49] While numerous activating agents have been used to synthesize DEL (such as HATU, EDCI, PyBOP), DMTMM is the most widely used activating agent as it shows impressive substrate tolerance.^[49,62,63] However, DMTMM is expensive, large excesses of are generally required (up to 10000 equiv), and large scale implementation is complicated due its instability in organic solution.^[64] In this context, the development of efficient coupling conditions of amino acids and peptides on DNA are of high importance both for the synthesis of therapeutic ON and to improve the preparation of DEL.

Recently, we reported a catalytic and sustainable approach for the direct coupling between an amine and a carboxylic acid using DABCO as catalyst and 2,4-dichloro-6-methoxy-1,3,5-triazine (DCMT) as substoichiometric activating agent.^[39] Considering the advantages of this catalytic method using cheap and non-hazardous reactants, we set out to explore its extension to the preparation of POCs both on solid support in anhydrous conditions and in aqueous conditions using in each case 2-chloro-4,6-dimethoxy-1,3,5-triazine (CDMT) and catalytic amounts of DABCO (Figure 1b).

Results and Discussion

Synthesis of POCs on CPG Beads

Our investigation began with the application on CPG-beads of our previously reported reaction conditions for practical supported synthesis.^[39] Optimization of the reaction was performed on a hexathymidine DNA sequence modified at its 5' extremity by an amino group introduced using a commercially available 5'-amino modifier phosphoramidite (ODN1) and Fmoc-Leucine as the acid partner (see ESI for details). The carboxylic acid was preactivated using our new CDMT/DABCO system prior addition on the CPG beads. Once the reaction fully optimized, the method was applied to various oligodeoxynucleotides (ODNs) and acid partners (Table 1), in particular single amino acids and peptides. According to our previous work, we found out that 1.1 equivalent of triazine and 10 mol% of DABCO towards carboxylic acids was sufficient for an efficient and complete activation (Table 1, entry 1). We observed excellent HPLC conversion with only 25 equivalents of activated acid. Amongst the side products, we were able to identify only one that contained an oligonucleotide, consequent to the attack of the primary amine to the triazine core instead of the carboxylic carbon. The amount of this triazine-oligonucleotide was never quantified but was negligible in most cases (<5%). These conditions were reproduced very efficiently on a heteropolymeric 12-mer, **ODN2** (Table 1, entry 2) to access the desired product with quantitative conversion and 64% isolated yield. When applied to a 21-mer, **ODN3** we found that 50 equivalents of the activated acid were required for full completion (Table 1, entry 3). Other single amino acids, such as Fmoc-protected Lysine and Arginine were also efficiently conjugated (Table 1, entries 4 to 8). Incorporation of a Fmoc protected amino acid on CPG-beads is convenient to obtain a fully deprotected POC as the Fmoc is removed during the final aqueous ammonia treatment required for the cleavage of the support. Boc deprotection of the lysine on CPG-beads, was achieved following reported conditions (TFA/DCM, Table 1, entry 6).^[57] An excellent yield was obtained, without noticing any degradation of the 12-mer. With these results in hand, applications of the reactions conditions to the dipeptide Fmoc-Pro-Val-OH, and the tripeptide Fmoc-Pro-Phe-Gly-OH yielded efficiently the corresponding conjugates. With **ODN2**, the corresponding POCs were obtained with 94% and 96% of conversion (Table 1, entries 10 and 11). Reaction of the same tripeptide and **ODN3** yielded 91% of conversion (Table 1, entry 12). To prove the efficiency of our method for the synthesis of bioactive POC, we applied our conditions to a phosphodiester analog of Fomivirsen, a synthetic 21-nucleotide antisense ODN indicated for the treatment of cytomegalovirus retinitis. The corresponding POC was obtained in a good 92% conversion and 34% isolated yield (Table 1, entry 13). Finally, when applied to a amphipathic hexapeptide, the Fmoc-VLRPPP-OH, monomer of the reported cell penetrating peptide SAP (Sweet Arrow Peptide),^[65] we were satisfied to observe that both **ODN1** and **ODN2** reacted smoothly to generate the respective POCs with excellent conversions (Table 1, entries 14 and 15). These examples

Table 1. Scope of the conjugation reaction on CPG beads using a 5'-amino-modifier.

$\text{Fmoc-(AA)}_n\text{-OH} + \text{H}_2\text{N-(CH}_2\text{)}_6\text{-(ON)-} \xrightarrow[\text{2. NH}_3 \text{ (aq. 30\%), 18 h, 50 }^\circ\text{C}]{\text{1. CDMT (X * 1.1 eq.)}} \text{H-(AA)}_n\text{-NH-(CH}_2\text{)}_6\text{-(ON)-3'}$ <div style="display: flex; justify-content: space-around; font-size: small;"> DABCO (X * 0.1 eq.) DiPEA (X * 2.2 eq.) </div> <div style="display: flex; justify-content: space-around; font-size: small;"> MeCN (0.5 mM), 15 h, r.t. </div>				
Entry ^[a]	Reactants Acid partner	ON ^[b]	Expected (Fmoc-deprotected) POCs Conv.(%)	Isolated yields (%) ^[c]
1		ODN1	Quant.	72 (30 nmol)
2		ODN2	Quant.	64 (27 nmol)
3		ODN3	Quant.	48 (20 nmol)
4		ODN1	Quant.	70 (29 nmol)
5 ^[d]		ODN2	95	62 (26 nmol)
6 ^[e]		ODN2	95	64 (27 nmol)
7		ODN1	Quant.	70 (29 nmol)
8		ODN2	98	60 (25 nmol)
9	Fmoc-Pro-Val-OH	ODN1	Quant.	71 (30 nmol)
10		ODN2	94	63 (26 nmol)
11	Fmoc-Pro-Phe-Gly-OH	ODN2	96	49 (21 nmol)
12		ODN3	91	36 (15 nmol)
13		ODN4 Fomivirsin-like ON	92	34 (14 nmol)
14	Fmoc-VRLPPP-OH	ODN1	95	65 (27 nmol)
15		ODN2	93	50 (21 nmol)
16		ODN2	98	52 (22 nmol)
17		ODN3	97	38 (16 nmol)
18		ODN2	98	46 (19 nmol)
19		ODN3	96	34 (14 nmol)

Abbreviations: CDMT: 2-chloro-4,6-dimethoxytriazine; DABCO: 1,4-diazabicyclo[2.2.2]octane; DiPEA: Diisopropylethylamine; Fmoc: Fluorenylmethyloxycarbonyl; Boc: *tert*-Butoxycarbonyl; Pbf: 2,2,4,6,7-Pentamethyl-2H-benzofuran-5-sulfonyl. [a] Conditions: Reactions were run on 42 nmol scale of oligonucleotide. Acid partner (X eq., 50 mM in MeCN), CDMT (X*1.1 eq., 50 mM in MeCN) and DABCO (X*0.05 eq., 5 mM in MeCN) were mixed in an Eppendorf vial and stirred at 25 °C at the thermoshaker. After 30 minutes, this preactivated solution was added in an Eppendorf vial containing the ON on CPG. Then DiPEA (X*2.2 eq., 100 mM in MeCN) was added to the reaction mixture and let 15 hours in Thermoshaker at 25 °C. Aqueous ammonia (1 mL) was added to the Eppendorf and after 18 h at 40 °C, the suspension was filtered, and the filtrate was lyophilized. Analysis by HPLC and MALDI-TOF-MS was done then a preparative RP-HPLC was performed to isolate the pure POC. [b] **ODN1**, X=25, 5'-NH₂-C₆-d(TTT-TTT-3'; **ODN2**, X=25, 5'-NH₂-C₆-d(TTC-TGA-TAC-ATT)-3'; **ODN3**, X=50, 5'-NH₂-C₆-d(TTA-GCT-TCT-TCA-CAT-TCG-ATT)-3'; **ODN4**, X=50, 5'-NH₂-C₆-d(GCG-TTT-GCT-CTT-CTT-GCG)-3'; [c] Obtained after reversed phase RP-HPLC; [d] Boc-ON; [e] Boc-OFF: (Boc deprotection by TFA, see SI for details).

demonstrated that SAP-oligonucleotide conjugates could be efficiently prepared on solid support and represent an interesting alternative compared to previously reported supported methodologies that required a late stage modification of ornithine to generate the arginine residue present in the SAP.^[66,67] In our case, protected and unprotected arginine was efficiently introduced on the oligonucleotide (Entries 7, 8, 14 and 15). Finally, to extend our method to other carboxylic acid partners, we performed the reaction on biotin and deoxycholic

acid with success on both **ODN2** and **ODN3** (Table 1, entries 16–19). It should be noted that we purified our POCs by reversed-phase HPLC. This resulted in a $\geq 95\%$ purity of the targeted oligonucleotides that is required for more demanding applications. It is therefore not surprising that despite excellent conversions and satisfying crude chromatograms, the recovery yields from oligo purification was 25–50% below of the synthetic yield.

Synthesis of POCs in Aqueous Solution

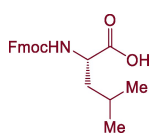
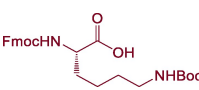
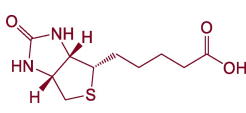
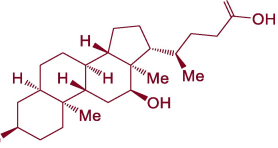
To broaden the application of our methodology, we considered the post-synthetic coupling strategy in aqueous solution. Optimization was run using **ODN1** on a 20 nmol scale and two different acids: Fmoc-Leu-OH, Fmoc-Val-Leu-Phe-Gly-OH. In both cases, optimized conditions were obtained by adding the activated acid in two separate portions (2* 125 eq.), see ESI for full details.

Once the conditions optimized, the scope and limitations of the reaction were studied (Table 2). In general, conversions and yields obtained in solution were slightly lower compared to the synthesis on solid support. As observed on solid phase, the

major side product is the triazine-ON, however, its amount stays below 10%. After coupling, Fmoc-protected POCs were treated with a solution of piperidine (10% in water) to achieve full deprotection. The incorporation of Fmoc-Leu on both **ODN2** and **ODN3** gave excellent conversions (Table 2, entries 1 and 2). We have also applied our conditions to a fully 2'-O-methoxy oligoribonucleotide 12-mer **ORN1** and showed that the coupling of Fmoc-Leu happened with good conversion (Table 2, entry 3). The same amino acid was also conjugated to fluorescently labeled **ODN5** and **ODN6** (6-FAM and Cy3 respectively, Table 2, entries 4 and 5).

Similar observations were made for the incorporation of a protected lysine on **ODN1** and **ODN2** (Table 2, entries 6 and 7).

Table 2. Scope of the conjugation reaction in aqueous media using a 5'-amino-modifier.

$\text{Fmoc-(AA)}_n\text{-OH} + \text{H}_2\text{N-(CH}_2\text{)}_6\text{-(ON)}^3 \xrightarrow[\text{MeCN/Borate buffer (1:1, 0.1 mM), 15 h, r.t.}]{\text{CDMT (2*125 * 1.1 eq.), DABCO (2*125 * 0.1 eq.), DiPEA (2*125 * 2.2 eq.)}}$ $\text{H-(AA)}_n\text{-NH-(CH}_2\text{)}_6\text{-(ON)}^3$ <p>then deprotection with 10% piperidine in water, 2 h, r.t.</p>				
Entry ^[a]	Reactants Acid partner	ON ^[c]	Expected (Fmoc-deprotected) POCs Conv. (%)	Isolated yields (%)
1		ODN2	97	39 (7.8 nmol)
2		ODN3	96	36 (7.2 nmol)
3		ORN1	88	30 (6.0 nmol)
4		ODN5	92	33 (6.6 nmol)
5		ODN6	94	40 (8.0 nmol)
6		ODN1	Quant.	42 (8.4 nmol)
7		ODN2	89	38 (7.6 nmol)
8 ^[b]	Fmoc-Pro-Phe-Gly-OH	ODN2	90	31 (6.2 nmol)
9 ^[b]		ODN3	88	29 (5.8 nmol)
10 ^[b]		ODN4 Fomivirsin-like ON	87	18 (3.6 nmol)
11		ODN1	91	25 (5.0 nmol)
12		ODN2	89	20 (4.0 nmol)
13 ^[b]		ODN2	93	48 (9.6 nmol)
14 ^[b]		ODN3	91	35 (7.0 nmol)
15 ^[b]		ODN2	93	44 (8.8 nmol)
16 ^[b]		ODN3	92	30 (6.0 nmol)

[a] Conditions: Reactions were run on 20 nmol scale of oligonucleotide. Acid partner (125 eq., 100 mM in MeCN), CDMT (137.5 eq., 100 mM in MeCN) and DABCO (12.5 eq., 10 mM in MeCN) were mixed in an Eppendorf vial and stirred at 25 °C at the Thermoshaker. After 30 minutes, this preactivated solution was added to the solution of ON in borate buffer (250 mM, pH 9.3, 100 μL) and then DiPEA (275 eq., 200 mM in MeCN) was added to the reaction mixture. After 2 hours stirring, another 125 equivalents of activated acid (same preparation as before) was added, and the reaction mixture was let 15 hours in Thermoshaker at 25 °C. Organic residues were extracted three times with 100 μL ethyl acetate. The aqueous phase was further lyophilized. Analysis by RP-HPLC and MALDI-TOF-MS was done then a preparative RP-HPLC was performed to isolate the pure POC; [b] Extra preactivated acid solution (see [a]) was added to the reaction mixture after 2 hours to ensure full conversion; [c] **ODN1**, 5'-NH₂-C₆-TTT-TTT-3'; **ODN2**, 5'-NH₂-C₆-d(TTC-TGA-TAC-ATT)-3'; **ODN3**, 5'-NH₂-C₆-d(TTA-GCT-TCT-TCA-CAT-TCG-ATT)-3'; **ODN4**, 5'-NH₂-C₆-d(GCG-TTT-GCT-CTT-CTT-GCG)-3'; **ODN5**, 5'-NH₂-C₆-TTT-TTT(6-FAM)-3'; T(6-FAM): 5-[N-(N-(Fluorescein-6-ylcarboxyl)aminoethyl)-3-acrylimido]-2'-deoxyUridine; **ODN6**, 5'-Cy3-TTT-TTT(C₆-NH₂)-3'; Cy3 : Cyanine-3, T(C₆-NH₂): 5-[N-(Aminoethyl)-3-acrylimido]-2'-deoxyUridine; **ORN1**, 5'-NH₂-C₆-U_{OMe}U_{OMe}C_{OMe}-U_{OMe}G_{OMe}A_{OMe}-U_{OMe}A_{OMe}U_{OMe}A_{OMe}U_{OMe}U_{OMe}-3'.

Conjugation with a tripeptide Fmoc-Pro-Phe-Gly-OH allowed us to prepare different POCs in good and similar conversions (Table 2, entries 8 to 10). The amphipathic hexamer, derived from a CPP, was coupled with both ODN1 and ODN2 in good yields, (Table 2, entries 11 and 12). To conclude on the scope, we extended this method to other carboxylic acid partners, such as biotin or deoxycholic acid, resulting in good conversions and yields to the corresponding ON conjugates (Table 2, entries 13 to 16).

The ability of this coupling reaction to incorporate efficiently single amino acids, prompted us to evaluate our conditions for iteration processes required for DEL synthesis. Using the optimized conditions developed in solution, a Fmoc-Phenylalanine was added on the 5' position of the amino-modified ODN2 (Figure 2). After coupling, the mixture is treated with piperidine (10% in water) to remove the Fmoc-protection. The resulting aqueous solution was then submitted to a NAP purification to remove the leftovers of small organic reagents, as well as the buffer salts. A second and third incorporation of Fmoc-Proline was then performed following the same procedure. The expected POC Pro-Pro-Phe-ON was obtained in a good 35% isolated yield after RP-HPLC purification that compares favorably with the direct coupling of a tripeptide to ODN2 (Table 2, entry 8), showcasing the versatility of our method to prepare POCs by iterative process that is a fundamental key point for its application on DEL's synthesis.

While the common strategies found in the literature to build a DNA encoded library involve DMTMM (from 40 equivalents to 10000),^[68] the use of DMTMM is not devoid of drawbacks, such as high price, safety and storage issues (44.60 €·g⁻¹ at Aldrich, storage at -20 °C),^[69] compared to CDMT (10.06 €·g⁻¹ at Aldrich, storage at room temperature).^[70] Moreover, all these methods to prepare POCs are often substrate dependent. For comparison, other coupling agents were evaluated (HATU, EDC/NHS, PyBOP and DMTMM) and the results confirmed the overall performance of our CDMT/DABCO method (SI, Tables S4 and S5). The newly reported conditions are appealing to replace DMTMM in such reactions, as well as replacing EDCI·HCl, HOAt, HATU for all reasons of price, safety and storage conditions. Therefore, our new method reported here expand the toolbox of catalytic methods for amide coupling reactions in the context of POCs' preparation as well as building DEL.

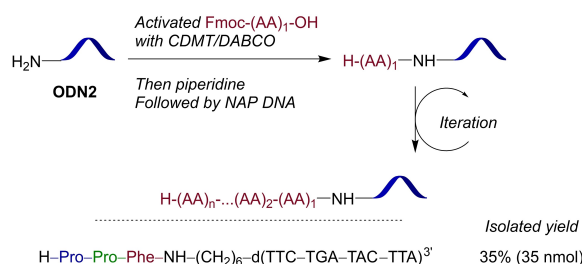


Figure 2. Iteration of the process.

Conclusions

In this work we have developed a new method to prepare peptide-oligonucleotide conjugates. The catalytic system CDMT/DABCO have proven to be very efficient both on-column conjugation in anhydrous media and in aqueous conditions. This method brings an alternative to the few methods that exist on solid support. To showcase its versatility, we have prepared various POCs, using peptides of different lengths (up to hexapeptide) and variously functionalized ON. Moreover, we have proven the possibility to perform iteration with this method, which is of high importance in DEL technology. Considering the high interest of POCs in hybrid nanomaterials and in therapeutic chemistry, we hope this work will contribute and help researchers to improve their synthesis by adding a new method in the toolbox of amide synthesis on oligonucleotides.

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Conflict of Interests

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: Amide · Peptide-Oligonucleotide Conjugates · Catalysis · Supported synthesis · Solution synthesis

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