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New Solid Support for the Synthesis of 3′-Oligonucleotide Conjugates through Glyoxylic Oxime Bond Formation

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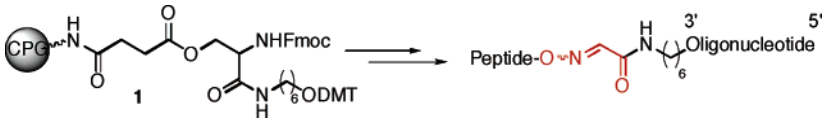
ABSTRACT



A novel solid support 1 was synthesized to incorporate glyoxylic aldehyde functionality at the oligonucleotide 3′-terminus. 6-mer and 11-mer oligonucleotide sequences containing 3′-glyoxylic aldehyde functionality were prepared by using this support. These modified oligonucleotides were coupled to reporters containing an aminooxy group to prepare oligonucleotide 3′-conjugates through glyoxylic oxime bond formation. The hydrolytic stability of a glyoxylic oxime linkage was also investigated.

Oligonucleotides (ODNs) have been extensively investigated for selective inhibition of gene expression,[1] DNA-based vaccines,[2] preparation of microarrays for diagnostic applications,[3] and design of nanostructures[4] with potential applications in electronic and/or photonic devices. However, certain intrinsic oligonucleotide properties (such as stability to degradation, cell uptake, and targeting, etc.) need to be further optimized to completely realize the full potential of these reagents. These intrinsic properties can be further improved by modifying oligonucleotides with molecules such as peptides,[5] carbohydrates,[6] lipids,[7] metal complexes,[8] and/or fluorophores. Thus, design and development of synthetic protocols for oligonucleotide conjugation has attracted considerable research interest. The most common approach used to prepare ODN conjugates involves separate preparation and purification of the oligonucleotide and the reporter followed by their solution-phase coupling. This is achieved by incorporating mutually reactive groups into each fragment, and the process leads to the formation of stable chemical linkages such as thioether,[9] disulfide,[10] oxime,[11] and hydrazone.[12] Recently, click chemistry[13] has also been investigated for ODN conjugation. Oxime linkages formed by the reaction of an aldehyde with an aminooxy group remain the most

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widely used chemical linkage for ODN conjugation. This is due to the fact that reactions leading to the formation of oxime bonds are chemoselective and give high coupling efficiency. Furthermore, these bonds are stable over a wide pH range. Oxime bond formation has been extensively used for the preparation of ODN conjugates with peptides,[14] carbohydrates,[15] and fluorescent probes.[16] It has been shown that ODN-glyoxylic aldehyde (α-ketoaldehyde)[17] is more stable to air oxidation and does not react with amino groups during ligation. These glyoxylic aldehyde functionalities are usually generated by periodate oxidation of a serine moiety and have been extensively used in protein engineering.[18] However, only a few methods have been reported in literature that can be used to prepare ODN 5′-conjugates through glyoxylic oxime bond formation.[17,19] To the best of our knowledge, there is no known method available for the preparation of ODN 3′-conjugates by a similar method.

Recently, a method to prepare peptide−oligonucleotide conjugates (POCs) through glyoxylic oxime linkage was reported from our laboratory.[19] The glyoxylic aldehyde linker was incorporated at the 5′ extremity of ODN by using a novel serine-containing phosphoramidite. The glyoxylic aldehyde function was generated by the oxidation of the serine moiety, and it was also shown that a glyoxylic oxime linkage is more stable than an aldoxime linkage at acidic to neutral pH. It was therefore decided to develop a protocol for the preparation of ODN 3′-conjugates through glyoxylic oxime bond formation. This is of significant interest because 3′-modified ODNs show greater resistance to nuclease activity compared to 5′-analogues. Furthermore, 3′-conjugation keeps the 5′-terminus free for ^{32}P-labeling by kinase, which is a very widespread technique used in molecular biology for DNA gel analysis.

We report, herein, a new and convenient procedure to prepare oligonucleotides modified with glyoxylic aldehyde at the 3′-terminus. This has been achieved by synthesizing a novel solid support **1** for ODN synthesis and modification (Figure 1). 6-mer and 11-mer oligonucleotide sequences modified with 3′-glyoxylic aldehyde group were prepared using this support. The efficiency of this procedure for 3′-conjugation was investigated by coupling these ODN sequences to aminooxy-containing peptides (sequences containing a RGD and NLS motif, respectively) and a fluorescein derivative. The arginine−glycine−aspartic acid (RGD) tripeptide motif is known to be a selective and powerful ligand for $\alpha_v\beta_3$ integrin receptors.[20] The NLS peptide is a nuclear localizing signal sequence with basic peptide APKKKRKVED derived from the simian virus 40 antigen. The hydrolytic stability of the 3′-glyoxylic oxime linkage was investigated and compared to the stability of the 3′-aldoxime linkage.

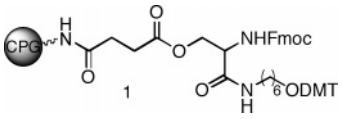
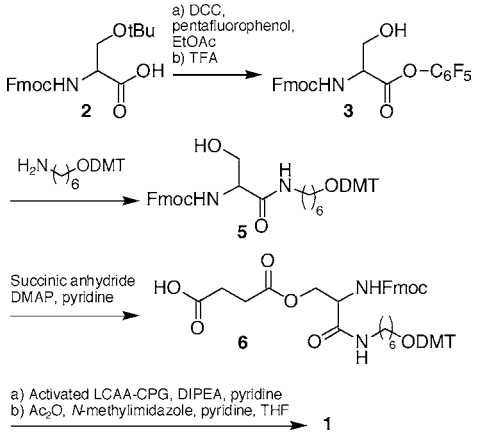


Figure 1. Solid support **1**.

The new long-chain alkyl amine-controlled pore glass (LCAA-CPG) solid support **1** was prepared from commercially available *N*-α-Fmoc-*O*-^{t}Bu-L-serine **2** in few chemical steps (Scheme 1). **2** was converted to pentafluorophenyl ester using pentafluorophenol/DCC. The ^{t}Bu protecting group was removed using 100% trifluoroacetic acid to obtain **3**. This on N-acylation with 1-*O*-dimethoxytrityl-6-amino-1-hexanol **4** gave compound **5**. The procedure to prepare protected amino linker **4** has been described earlier.[21] It should be mentioned that β-hydroxy protected *N*-α-Fmoc-*O*-^{t}Bu-L-serine **2** was used as the starting material instead of β-hydroxy unprotected *N*-α-Fmoc-L-serine because the latter compound showed significant side reactions on either conversion to pentafluorenyl ester or acylation with **4** under standard peptide coupling conditions. The synthon **5** was anchored onto the solid support by using the "classical" succinyl linker. The succinyl linker was attached at the β-hydroxyl function of **5** via an esterification reaction with succinic anhydride to obtain **6**. The acid **6** was finally attached to the solid support by reaction with the amino

Scheme 1. Preparation of Solid Support **1**



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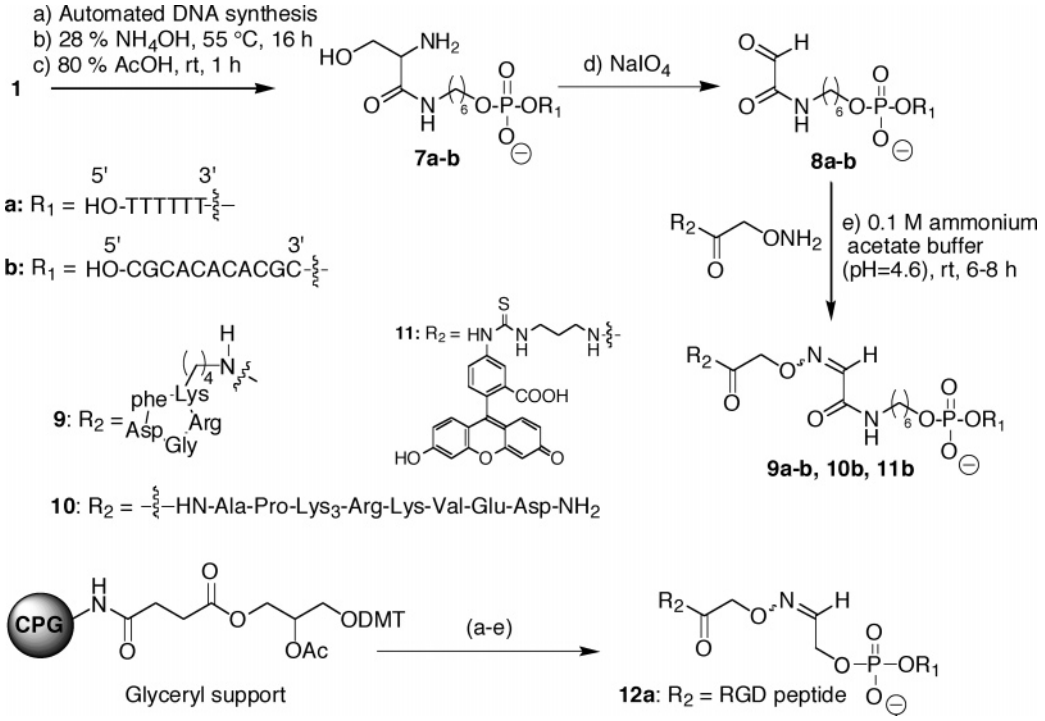
Scheme 2. Synthesis of Peptide−Oligonucleotide Conjugates Using Support **1** or Commercially Available Glyceryl CPG Supports



moiety present on the support using a standard procedure.[22] Finally, unreacted amino groups on LCAA-CPG were capped with acetic anhydride. The loading of the support was found to 35 μmol/g as estimated from a dimethoxytrityl cation UV assay. The advantage of using support **1** is that the Fmoc group can be easily removed by an ammonia treatment which is employed for nucleobase deprotection in standard DNA synthesis protocols. Besides, the C6 linker should be sufficient to minimize steric hindrance between the ODN and the reporter.

The support **1** was first used to prepare 6-mer **9a** and 11-mer **9b** oligonucleotide sequences (Scheme 2). ODN sequence **9a** is a simple hexathymidylate. This was used to optimize the automated synthesis on support **1**, the oxidation procedure, and the conjugation protocol. Oligonucleotide elongation was performed by using the standard phosphoramidite protocol on a 1 μmol scale. The ODNs were cleaved from support and released into the solution by treatment with ammonia.

The nucleobase deprotection and simultaneous removal of the Fmoc group were accomplished by keeping the ammonia solution at 55 °C for 16 h. The ODN sequences so prepared were analyzed by RP-HPLC on a C_{18} column. The HPLC profile showed the presence of a major ODN product along with a minor amount of truncated sequences. The 5′-DMT group was removed by treatment with 80% aqueous acetic acid to obtain the oligonucleotide **7a** containing the free serine at the 3′-end. The serine moiety was successfully oxidized by using excess aqueous sodium periodate to obtain ODN 3′-glyoxylic aldehyde **8a**. The oxidative cleavage was found to be clean, leading to the exclusive formation of ODN 3′-glyoxylic aldehyde. As reported earlier, **8a** was found to be stable for months at −20 °C.

The suitability of an ODN-glyoxylic aldehyde for 3′-conjugation was investigated by synthesizing peptide oligonucleotide conjugate **9a**. The ODN−glyoxylic aldehyde **8a** was reacted with an aminooxy-containing RGD peptide[23] in 0.1 M ammonium acetate buffer at room temperature. The reaction was carefully monitored by RP-HPLC. The HPLC profile showed the exclusive formation of conjugate **9a** in about 8 h (see the Supporting Information). The peptide−ODN conjugate (POC) **9a** containing the 3′-glyoxylic oxime linkage was obtained in satisfactory yields after HPLC purification. The 11-mer ODN-glyoxylic aldehyde **8b** was prepared by using a similar procedure. It should be mentioned that the ODN sequence **8b** is a heterooligonucleotide containing different types of nucleotides, unlike **8a**. This was used to prepare peptide−ODN conjugate **9b** as described above. The efficiency of the method is further illustrated by coupling the ODN-glyoxylic aldehyde **8b** to a nuclear localizing signal (NLS) peptide sequence functionalized with an aminooxy function[23] as well as to a fluorescein probe[24] to afford the conjugates **10b** and **11b**, respectively.

The ODN derivatives and conjugates prepared herein were characterized by ESIMS analysis. The observed molecular

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weights were found to be in excellent agreement with calculated values (Table 1).

Table 1. ESIMS Data[a]

compound	*m/z* calcd	*m/z* found
7a	2027.4	2027.4
7b	3534.7	3533.3
8a	1996.4	1996.7
8b	3503.6	3502.6
9a	2654.7	2654.8
9b	4162.0	4160.5
10b	4755.4	4753.7
11b	4021.8	4020.2
12a	2541.6	2542.0

[a] Analysis was carried out in the negative mode. $CH_3CN/H_2O/Et_3N$ (50:50:2, v/v/v) was used as the eluent at a flow rate of 8 μL min^{-1}.

The hydrolytic stability of conjugate **9a** containing the glyoxylic−oxime bond was investigated. A control conjugate **12a** containing an aldoxime linkage was also prepared for comparison.[23] Purified conjugates **9a** and **12a** were incubated (10^{-5} M) in phosphate buffer solutions with pH values adjusted to 4, 7, and 9. The percentage of hydrolysis of the two linkages was estimated by RP-HPLC analysis after 24, 48, and 120 h. The data obtained are collected in Table 2, and it clearly shows that the glyoxylic oxime conjugate is more stable under acidic to neutral conditions. Only 5% hydrolysis was observed in conjugate **9a** (120 h, pH 4 and 7), whereas conjugate **12a** showed 31% hydrolysis only in 24 h under similar conditions (pH 4). On the contrary, conjugate **12a** was found to be more stable under alkaline conditions. **12a** showed about 6% hydrolysis at pH 9 (120 h), and conjugate **9a** showed 36% hydrolysis under similar conditions. The results obtained herein support our earlier stability studies reported for ODN 5′-conjugates.[19]

Thus, a new and convenient procedure has been developed for the synthesis of 3′-oligonucleotide conjugates through the formation of glyoxylic oxime bonds. This has been achieved by using a novel solid support **1** for ODN synthesis. Support **1** was conveniently prepared from a commercially available serine derivative in few steps. The support incorporates a masked glyoxylic aldehyde precursor at the 3′-ODN terminus. Mild oxidation of this precursor leads to the formation of ODN containing a 3′-glyoxylic aldehyde. ODN-glyoxylic aldehydes undergo a chemoselective coupling reaction with the aminooxy group to give ODN 3′-conjugates. The glyoxylic oxime bonds showed higher stability than aldoxime bonds at acidic to neutral pH but lower stability at alkaline pH. The procedure developed herein can easily be extended to the preparation of various other classes of oligonucleotide conjugates.

Table 2. Percentage of Hydrolysis in Conjugates **9a** and **12a** at Different pH (37 °C)

	% hydrolysis[a]					
conjugate	**9a**			**12a**		
pH	4.0	7.0	9.0	4.0	7.0	9.0
24 h	<3	<3	<5	~31	~9	<6
48 h	<3	<3	~13	~45	~10	<6
120 h	<3	<5	~36	~51	~13	<6

[a] Percentage of hydrolysis was estimated from the area under the peak in the HPLC chromatogram (260 nm).

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Supporting Information Available: Experimental procedures, RP-HPLC profiles and ESIMS spectra, and T_m and CD data. This material is available free of charge via the Internet at http://pubs.acs.org.

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