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# Guanine rich oligonucleotide–amino acid/peptide conjugates: preparation and characterization

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**Abstract**—Covalent addition of aminoacyl or peptidyl groups could improve the performance of RNA/DNA molecules either in catalysis or in other attributes such as their ability to interact with membranes. This can explain the transformation of the RNA world first into a peptidyl-RNA world and eventually into a RNA–protein world. Subsequently, the emergence of DNA as more stable storage of genetic information than RNA finally gave shape to present day DNA–RNA–Protein world. For this reason, this paper reports a stepwise study, of the direct incorporation of amino acid/peptide onto synthetic amino modified deoxyoligonucleotide. The amino acid/peptide has been covalently linked to the primary amine of amino modified oligonucleotide, which is separated from the oligomer by a spacer arm of 10 atoms. The yield depends on the presence of bulky amino acid side chains that possibly hinder the attack of incoming nucleophile. On the other hand, the use of relatively high concentrations of EDC has improved the conjugation yield remarkably and complete conjugation can be achieved within few hours. Such a facile incorporation of amino acids or peptides may help oligonucleotides with greater cell membrane permeability, stability and reactivity. 

## 1. Introduction

In recent years, the number of naturally occurring RNA enzymes 'ribozymes' performing variety of functions such as DNA cleavage[1] self-slicing[2] polymerization of RNA molecules and its units[3] have been reported. Mostly, these reactions are known to occur at the phosphorus centers. However, the catalytic activity of ribozymes can greatly be improved at other than phosphorus centers through combinatorial methods 'in vitro selection' and 'in vitro evolution'.[4] Moreover, these methods are now successfully applied to isolate DNA enzymes or 'deoxyribozymes',[5–7] for example, imidazole containing DNA can efficiently cleave an RNA substrate.[8] Although DNA is regarded as unsuitable as a nucleic acid enzyme due to absence of diverse functional groups, but with the rational design and engineering of deoxyribozymes may increase the catalytic capabilities several-fold by single or multiple attachments of versatile amino acids/peptides having more functionalities.

Also, such covalent conjugates of amino acids/peptides and nucleic acids might be a crucial link between both the RNA and protein worlds and could possibly have played a key role in the transition from a primordial RNA world to a present day protein world. It has been proposed that oligoribonucleotides or polyribonucleotides modified with amino acids would enhance the catalytic capabilities of ribozymes or deoxyribozymes, and may have led the way to the participation of peptide sequences in biological systems.[9,10] Although, RNA with its 2′ hydroxyl group is a more appropriate candidate to be a catalyst, however, the synthesis of more efficient DNA enzymes is in progress and are recommended as nucleic acid enzymes rather than RNA enzymes.[11] Furthermore, DNA enzymes are easier to prepare and less sensitive to chemical and enzymic degradation.[7,12]

Amino acid–nucleic acid conjugates are therefore of both fundamental and of applied significance. The derivatization of nucleic acid with the amino acids or peptides may provide a means to improve the function of an antisense oligonucleotide for application as potential therapeutic agents. Furthermore, such modifications are expected to increase cellular uptake of oligonucleotides and increase resistance to nucleases.

The synthesis of oligonucleotides–amino acids/peptide conjugates can be achieved by derivatization at a monomeric stage or by anchoring a functional group to oligonucleotide that consists of modification with as affinity reagent. Both approaches are quite common in practice; however, the post assembly functionalization approach to prepare oligonucleotide–amino acid/peptide conjugate has been adopted in this study as it allows rapid synthesis of conjugates and their analysis. The desired ligand can be attached at either of the terminal positions (5′ or 3′ end)

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of the phosphate moiety or at an internal position such as the base sites, backbone sites or sugar sites. Phosphate and ribose modified oligonucleotide analogues have shown high resistance to different nuclease activity and are also known to have higher biological activity in various systems. The base site, in particular C-5 position of pyrimidine is an important site for internal modification as it remains in the major groove of duplex without interfering with hydrogen bonding.[13] A range of chemical moieties including biotin, fluorescence labels, and pyrene sulfonates have been conjugated to this site.[14] Thus far, numerous linker groups have been reported to be attached to different sites of nucleotides[15,16] and have been used successfully as DNA probes for monitoring DNA conformation, kinking of DNA, and four way junctions.[17–20]

The objective of this paper is to report a simple and easy method for the covalent linking of amino acids and peptides to oligonucleotides having one modified thymidine nucleoside, T* that bears a reactive amino group on the C-5 position of pyrimidine. The presence of an amino group on the alkyl linker arm of a modified oligonucleotide allows the latter to be converted in to different reactive groups and labels. This paper investigates the carbodiimide mediated covalent linking of amino acids and peptides to oligonucleotides through an amino group derivatized on a modified thymidine. The ligation to amino acid or peptide is rapid and site specific, forming a stable amide bond. By covalently linking hydrophobic amino acids at one or two sites of the oligomer, the respective hydrophobicity of oligomer can be increased. The reaction was carried out using fully protected or partially protected amino acid/peptide with oligonucleotide in 50% DMF at pH 7.0. Characterization of the product was carried out by anion exchange HPLC and MALDI-TOF-MS.

## 2. Results and discussion

The origin of the genetic code and translation has been one of the major transition steps in the evolution. The discovery of catalytic RNA has suggested that RNA might have had both coding and catalytic functions prior to the development of genetic code and proteins. The linking of amino acids or small peptides to nucleic acids has been assumed to have a significant role in some form, in the metabolism of organisms in the RNA world, prior to utilization of these links in the protein synthesis.[9] In another approach, some researchers suggested that free amino acids could serve as cofactors for ribozymes and thus may have increased the versatility of nucleic acids.[12,22] Nucleic acid–peptide conjugates thus have both fundamental and applied significance; therefore, a systematic study on covalent attachment of amino acids, especially the hydrophobic amino acids was undertaken. Such attachments are expected to improve the absorption of oligonucleotides into biological membranes and enable action as DNA enzymes.

One approach for the covalent attachment of any label is to modify chemically the heterocyclic base of nucleotide that can provide a functional group for subsequent labeling. In the present study, modified thymidine with an alkyl linker arm bearing an amino group at C-5 position was used for

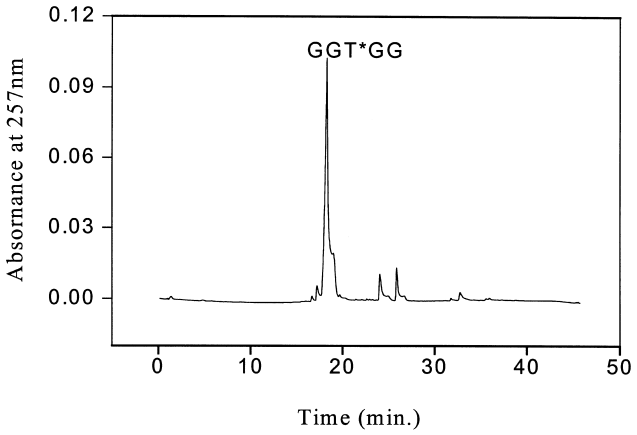



**Figure 1.** Anion exchange HPLC elution profile of GGT*GG at pH 7.0.

conjugating the amino acids and peptides to the oligonucleotide. The specific base sequence used in the present study is GGT*GG. This guanine rich sequence was selected as it is of special interest to many workers. These sequences are known to form a complex folded structure, DNA tetraplexes.[23] In the initial conjugation studies, a small oligomer GGT*GG, where the modified T* is 2′ deoxyuridine with a linker arm of 10 atom spacing attached at the C-5 position, was used. Shorter oligomers have known advantages over the longer ones; short oligomers are easy to prepare; further, their purification is much easier than that of longer ones.

The bioconjugation of oligonucleotides with peptides or other labels has been investigated by a number of research workers using chemistry of different functional groups or attachments or key position of nucleotides. Covalent attachment of particular group can be performed both prior to[24,25] and subsequent to[26–28] the incorporation of modified nucleoside into oligonucleotide. Conjugation of lysine and histidine initially to the 2′ deoxyuridine and subsequently its incorporation into oligonucleotides at internal position during solid phase synthesis was reported by Bashkin and coworkers[29] and recently, a DNA sequence modified at 5′ end was synthesized on a solid support followed by its coupling with photocleavable peptide was reported by Olejnik et al.[30] The covalent attachment of peptides to oligonucleotides has been reported from an antisense viewpoint and such attachments have facilitated the cellular uptake of oligomers. Cationic polylysine[31,32] and hydrophobic polytryptophan[33] attachments have significantly enhanced the penetration of oligomer into cells. Also, attachment of peptides to the 3′ end of oligonucleotide has been claimed to increase oligomer stability to the most ubiquitous exonucleases, particularly 3′ exonucleases.[34–36]

Coupling of the amino acid/peptide with the oligonucleotide was carried out by activating a carboxyl group of amino acid by a coupling reagent 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, EDC. The reaction between the carboxyl group of the amino acid and EDC gives a reactive intermediate *O*-acylisourea. Further addition of nucleophile yields a stable peptide bond product. Conjugation reactions were performed by adding a protected amino acid or peptide to EDC in DMF, followed by addition of the oligonucleotide

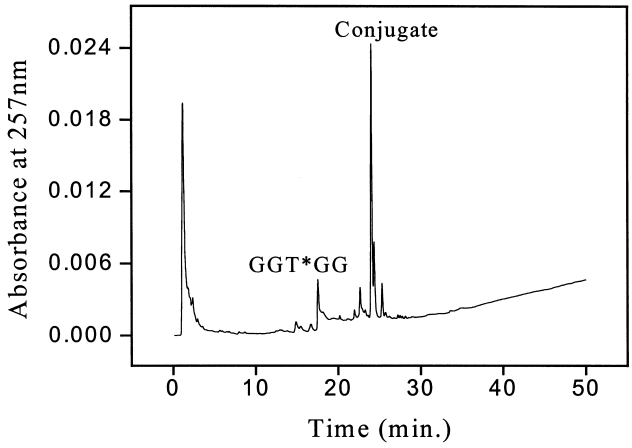



**Figure 2.** Anion exchange HPLC elution profile of GGT*GG–alanine-acetyl conjugate reaction mixture after 24 h at pH 7.0.

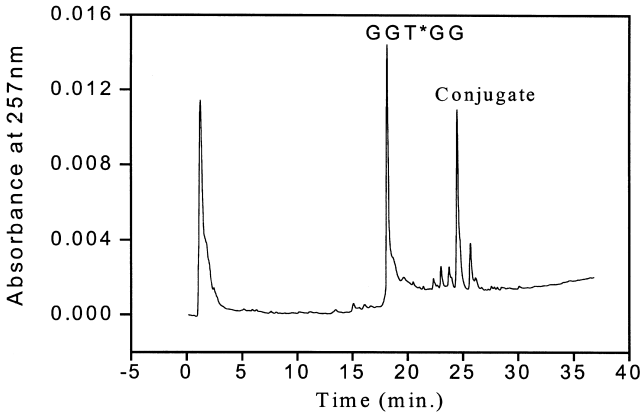



**Figure 4.** Anion exchange HPLC elution profile of GGT*GG–valine-acetyl conjugate reaction mixture after 24 h at pH 7.0.

and a buffer of the desired pH. Conjugation of oligonucleotide with amino acid or peptide was followed by anion exchange or reverse phase HPLC using a photodiode array detector and Millennium software. The HPLC elution profile and a typical UV spectrum of the oligonucleotide or conjugate can be viewed simultaneously by using this software. In the first 20 min of GGT*GG–alanine-acetyl conjugation reaction at pH 7.0, the yield of product was 85.80%, after 1 h 90%, after 2 h 91% and after 24 h 95.46%. It is obvious that the reaction is almost complete within the first 20 min of the reaction; however, to obtain the maximum yield of conjugate product, reactions were always carried out for 24 h.

In order to improve the yield of conjugate, a systematic study of the effect of pH, EDC and amino acid concentration was also carried out and optimum conditions were used for further studies of the conjugation reaction. The oligonucleotide–alanine-acetyl conjugation reaction was carried out over a wide pH range, 5.5–8.5 and was found to be highly pH dependent.[10] It was observed that at lower pH, the coupling yield was quite low and that it was profoundly increased with an increase in pH. It has been reported that amide bond formation is around 35–40% with the amine groups of the nucleotide bases rather than the intended linker in presence of EDC.[37] However, under the present experimental conditions, side reaction of amine bases was observed to be negligible, 2–5% as deduced from HPLC chromatogram (Figs. 1–7). This is probably due to the chemical inertness of aryl amines on heterocyclic compared with the aliphatic primary amine. Another possibility is that the linker arm makes the primary amine more accessible than amino groups on heterocyclic bases. The effect of EDC concentration on the conjugation reaction of GGT*GG–alanine-acetyl was studied by varying the concentration of EDC. The conjugation yield was also found to increase with increase in EDC concentration. Unless and otherwise stated, in most of the cases, the amino acid/peptide concentration was always considerably in excess of that of oligonucleotide because it was found that conjugation reaction was negligible at lower concentrations of amino acids.

Conjugation of amino acids to oligonucleotide can be achieved by using coupling agents like carbodiimides. They are widely used for effective modification and cross-linking of proteins and in peptide synthesis. Carbodiimides have also been suggested as a primordial coupling agent and reported to have facilitated the synthesis of biopolymers in aqueous solution.[38] Recently, carbonyl diimidazole (CDI) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were used as activating agent for the polymerization of β amino acids in aqueous solution.[39] We, therefore, have

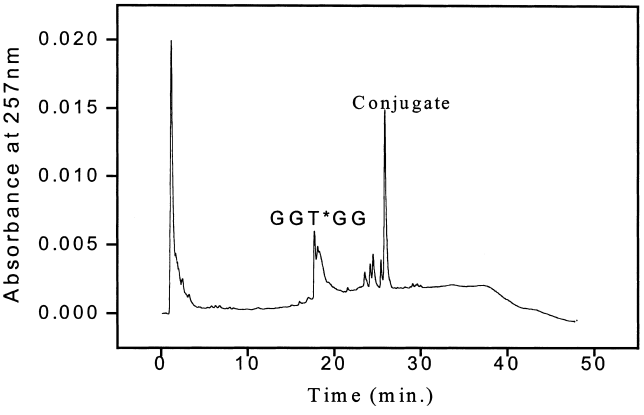



**Figure 3.** Anion exchange HPLC elution profile of GGT*GG–phenyl-alanine-acetyl conjugate reaction mixture after 24 h at pH 7.0.

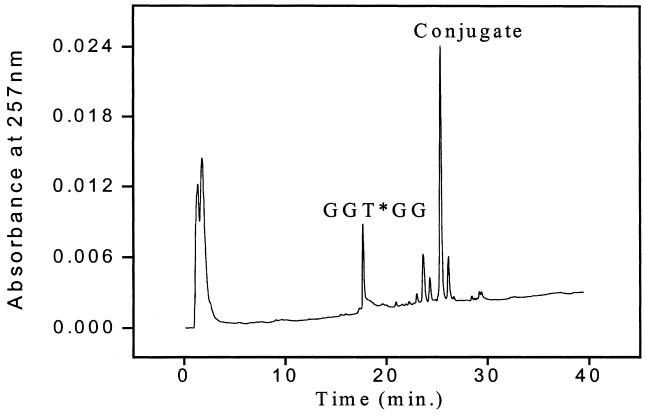



**Figure 5.** Anion exchange HPLC elution profile of GGT*GG–methionine-acetyl conjugate reaction mixture after 24 h at pH 7.0.

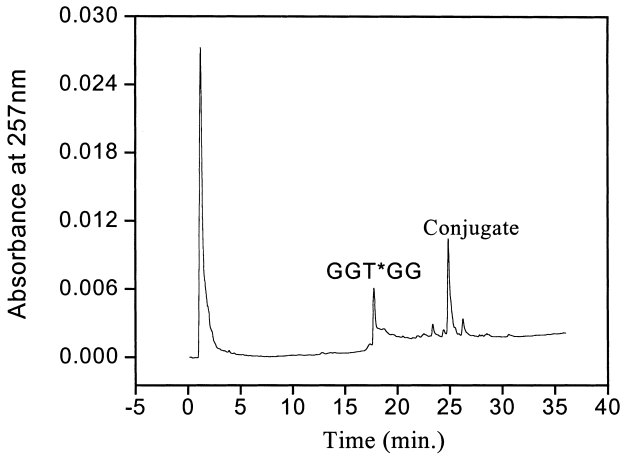



**Figure 6.** Anion exchange HPLC elution profile of GGT*GG–glycine-histidyl-acetyl conjugate reaction mixture after 24 h at pH 7.0.

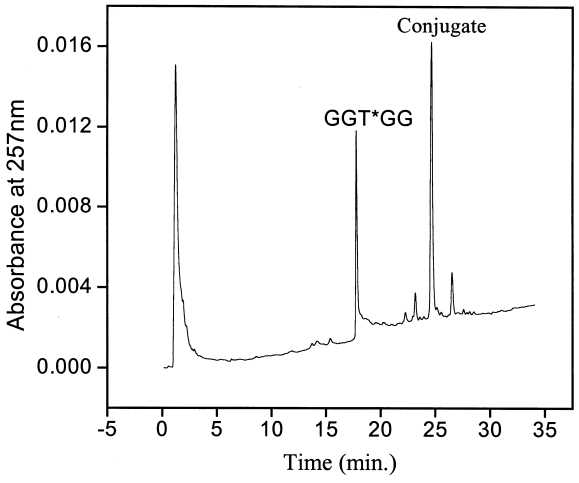
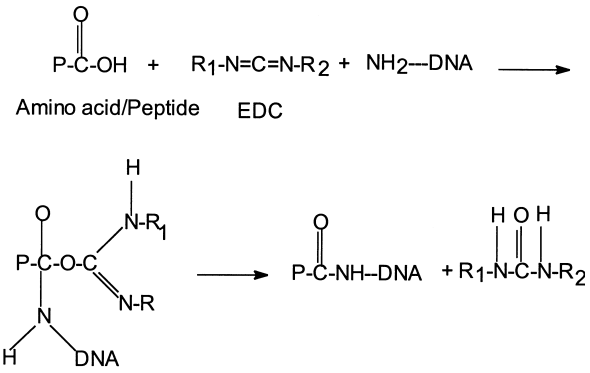



**Figure 7.** Anion exchange HPLC elution profile of GGT*GG–alanine-histidyl-acetyl conjugate reaction mixture after 24 h at pH 7.0.

also studied coupling efficiency of four commonly used conjugating agents EDC, DCC, CMC, and CDI on conjugation of GGT*GG and acetyl-alanine in detail to improve the overall yield of conjugation product. Under identical




**Scheme 1.**

experimental conditions, CMC did not show clear product formation while in case of DCC, amount of product formation was inconsistent in repeated sets of experiments. CDI gave only 60% yield; however, EDC has shown maximum coupling efficiency of 95%. Amongst four conjugating agents studied, EDC was the best coupling agent and thus was selected for all conjugation reactions.

Among the 12 acetyl amino acids used for the conjugation reaction of GGT*GG, acetyl-histidine, acetyl-cysteine and acetyl-serine did not show any reaction. Although the exact reasons for this lack of conjugation with these three amino acids remain to be defined, it is known that their side chains in the unprotected form could react with carbodiimide.[40] This has been attributed to the presence of strongly nucleophilic groups like imidazole sulfydryl and hydroxyl in the side chain of histidine, cysteine and serine respectively. The nucleophilic character of imidazole, sulfydryl and hydroxyl groups is quite pronounced and cannot be ignored in the conjugation reaction. In order to support the above reasoning, the nucleophilic side chain of imidazole group of histidine was masked with a suitable protecting group in addition to $N_\alpha$ amino group. Thus, $N_\alpha$-*t*-boc-im-benzyl-histidine was used in a conjugation reaction. A remarkably high conjugation yield was obtained when this fully protected amino acid was used in the conjugation reaction and confirmed the interference of side chain nucleophilic groups. However, conjugate products of $N_\alpha$-*t*-boc-*S*-benzyl-cysteine and $N_\alpha$-*t*-boc-*O*-benzyl-serine could not be

**Table 1.** HPLC and mass analysis of GGT*GG and its conjugates. Reaction conditions: [oligonucleotide]=0.1 mg/ml; [amino acid/peptide]=12.5 mM; [EDC]=37.5 mM; pH=7.0. MALDI-MS analysis of Sep-Pak purified conjugates was carried out in HPA matrix using linear positive mode

| Conjugate | Yield (%) | Retention time | Observed M.Wt. | Calculated M.Wt. |
|---|---|---|---|---|
| GGT*GG | * | 18.20 | 1712.00 | 1713.30 |
| Ac-Asp | 93.00 | 28.25, 29.11 | 1869.20 | 1870.40 |
| Ac-Gly | 98.10 | 26.84 | 1813.00 | 1812.30 |
| Ac-Ala | 95.46 | 24.35 | 1827.00 | 1826.30 |
| Ac-Met | 81.17 | 25.35 | 1884.40 | 1886.30 |
| Ac-Val | 47.43 | 24.47 | 1853.00 | 1854.30 |
| Ac-Leu | 61.00 | 24.60 | 1865.00 | 1863.30 |
| Ac-Tyr | 72.24 | 27.92 | 1917.20 | 1918.30 |
| Ac-Phe | 93.52 | 25.87 | 1904.00 | 1902.30 |
| Ac-Trp | 81.00 | 29.60 | 1938.60 | 1938.30 |
| Ac-His-Gly | 69.10 | 24.90 | 1947.00 | 1949.50 |
| Ac-His-Ala | 66.00 | 24.65 | 1962.10 | 1962.50 |
| *N*-*t*-Boc-im-His | 83.74 | 25.80 | 2038.30 | 2040.30 |
| *N*-*t*-Boc-*O*-Cys | * | * | 2004.30 | 2006.50 |
| *N*-*t*-Boc-*S*-Ser | * | * | 1990.10 | 1990.30 |

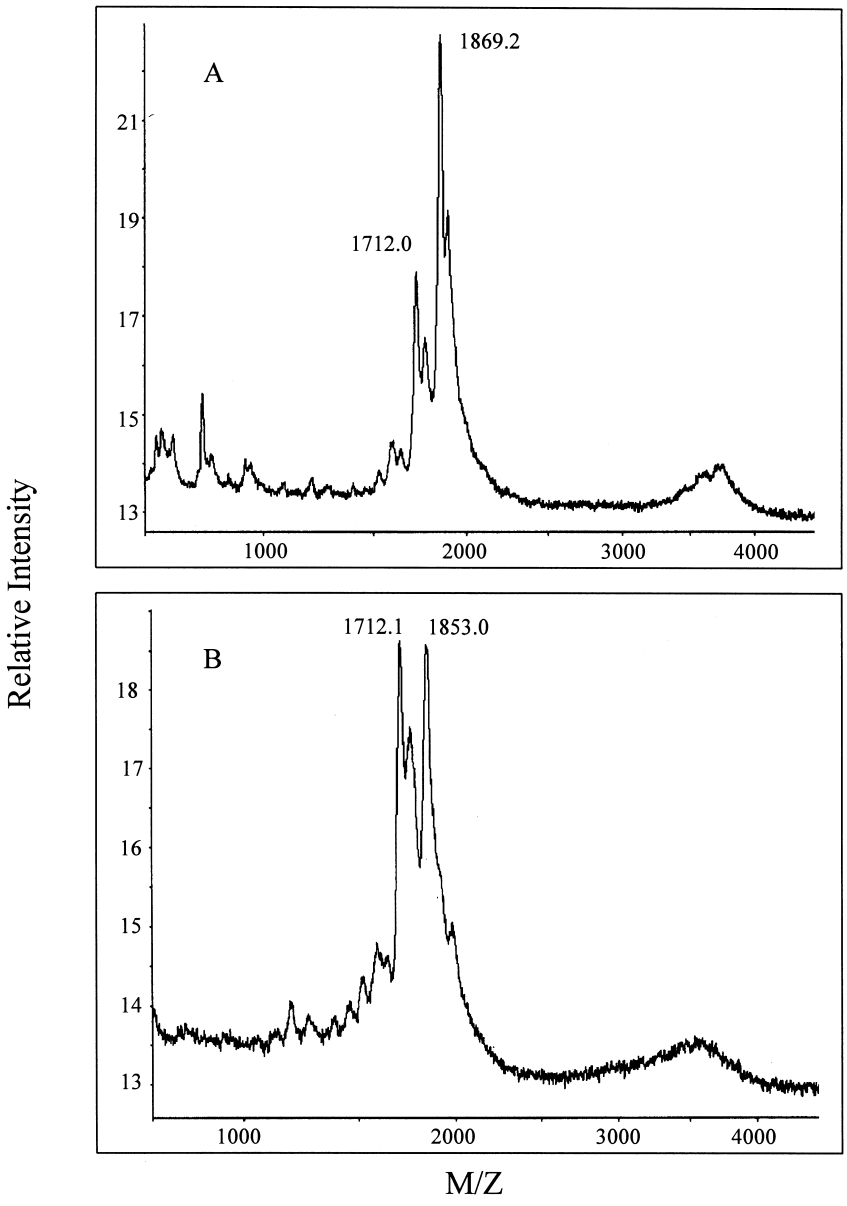



**Figure 8.** MALDI mass spectra using HPA as matrix in the linear positive mode. Additional mass peaks such as $[M+2H]^{++}$, $[2M+H]^{+}$ or depurination peaks were sometimes observed. A=GGT*(Asp-Ac)GG; B=GGT*(Val-Ac)GG.

identified by anion exchange HPLC but their formation was confirmed by MALDI mass spectrum analysis.

In another approach, we used acetyl-dipeptides of histidine with glycine and alanine in the conjugation reaction for two reasons, viz. to introduce hydrophobicity close to the hydrophilic histidine residue and to decrease the strong nucleophilic effect of side chains of histidine. When dipeptides of histidine, acetyl-histidyl-glycine and acetyl-histidyl-alanine were used in the conjugation reaction, the nucleophilic effect of imadazolium reduced drastically and the conjugation yields were observed to be around 69 and 66%, respectively (Figs. 6 and 7). Therefore, the negative effects of the unprotected His side chain could be partially overcome when these residues were placed one amino acid residue away from the conjugation site.

For comparison, the N-terminus amino group is protected by an acetyl group in all these amino acids and peptides. Overall results have indicated that the coupling of oligomer in 50% DMF was best achieved at higher pH and by employing higher amino acid and EDC concentrations. Thus, for conjugation reaction to occur between GGT*GG and amino acids/peptides, we have selected pH 7.0 and an EDC concentration three times higher than that of amino acid/peptide. With the above stated experimental conditions, the values obtained for percent conjugation, retention time and mass analysis for all amino acids/peptides are compiled in Table 1. Scheme 1 shows the possible conjugation of amino acid/peptide with oligonucleotides.

More interestingly, it has been observed that the conjugation yield of GGT*GG with each amino acid is dependent on the presence of bulky side chains of the amino acid, which is quite consistent with the reaction mechanism. Among the 12

amino acids used in the present studies, the maximum yield was obtained for glycine and then alanine followed by other amino acids. Quite precisely, the presence of bulky alkyl or aryl group such as methyl, dimethyl or phenyl on the side chain of amino acid tends to hinder the attack of incoming nucleophile thereby reducing the conjugation yield. Thus, the percentage of conjugation yield and the presence of bulky side chain on the amino acid can be correlated as follows: acetyl-glycine>acetyl-alanine>acetyl-phenyl-alanine>acetyl-leucine>acetyl-valine.

Furthermore, the conjugation yield of acetyl-tyrosine was observed to be less than acetyl-phenylalanine due to the presence of the hydroxyl group; a nucleophilic group on phenyl ring. However, the hydroxyl group on acetyl tyrosine is not as strong as in the case of acetyl-serine which totally failed to conjugate to GGT*GG. A similar explanation can be extended for acetyl-cysteine and acetyl-methionine and thus the conjugation product was obtained in the case of acetyl-methionine but not for acetyl-cysteine (Table 1).

MALDI-TOF mass spectrometers function on the principle of ionization of crystalline matrix in which the desired sample is embedded. Although a number of matrices have been tried for oligonucleotides,[41,42] however, 3-hydroxy-picolinic acid has been the most commonly used and performs well. MALDI mass spectra of small DNA oligomers have shown quite variable sensitivities depending upon base composition. Oligodeoxythymidylic acid $d(T)_{10}$ yields excellent quality spectra but other oligomers like $d(C)_{10}$ have shown much weaker spectra, and under identical experimental conditions, no results were obtained for $d(A)_{10}$ and $d(G)_{10}$.[43] Another factor that affects MALDI mass spectra is the presence of basic amino acid or peptide. Zhu et al.,[44] have reported that basic amino acids such as lysine and histidine strongly enhance MALDI signals in the positive-ion mode.

In our present studies, reasonably good spectra for all oligonucleotides and their conjugates with amino acids or peptides were obtained in positive/negative mode. More accuracy in mass analysis was observed in positive mode for smaller oligonucleotides like GGT*GG whereas negative mode was found to be a much better option for higher oligomers. Commonly, MALDI mass analysis of conjugates was carried out after Sep-Pak purification and was found to be quite accurate with an error limit of 0.01–0.5% after internal standard correction. Results of MALDI mass analysis of all conjugates are presented in Table 1 and MALDI mass spectra of conjugates have been presented in Fig. 8.

In conclusion, an efficient method for synthesis of oligonucleotide–amino acid/peptide conjugates using carbodiimides as activating agents has been developed. These covalently linked conjugates were purified by Sep-Pak reserve phase C18 cartridges and well characterized by HPLC and matrix assisted laser desorption spectrometry. These conjugates could underpin various biotechnological applications, besides playing a vital role in the prebiotic development of life forms. Furthermore, it is expected that such nucleotidyl-peptides conjugates will enhance capabilities of oligonucleotides for catalysis or hydrophobic interaction with membranes and other chemical structures. This method of post assembly attachment of pendant amino acid or peptides to oligomers can further be extended to higher oligonucleotides with multiple attachment sites.

## 3. Experimental

1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), *N*,*N*′-dicyclohexylcarbodiimide (DCC), 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-tolunesulphonate (CMC), 1,1′-carbonyldiimidazole (CDI), protected amino acids such as *N*-acetyl-glycine, *N*-acetyl-L-alanine, *N*-acetyl-L-phenylalanine, *N*-acetyl-L-valine, *N*-acetyl-L-leucine, *N*-acetyl-L-aspartic acid, *N*-acetyl-L-tryptophan, *N*-acetyl-L-tyrosine, $N_{\alpha}$-acetyl-L-histidine, *N*-acetyl-L-methionine, *N*-acetyl-L-leucine, $N_{\alpha}$-*t*-boc-*S*-benzyl-cysteine, *N*-*t*-boc-*O*-ben-Serine and $N_{\alpha}$-*t*-boc-im-benzyl-L-histidine, were purchased from Sigma Chemicals. The matrix, 3-hydroxypicolinic acid (HPA) used for MALDI-MS analysis was from Aldrich. The peptides, *N*-acetyl histidinyl-glycine, and *N*-acetyl-histidinyl-alanine were obtained from PeptidoGenic Research, Livermore, California. Modified oligomer GGT*GG, was purchased from Glen Research Laboratories. The modified T* in oligonucleotide in these oligonucleotides is 2′deoxyuridine and after deprotection, the primary amine on amino modified C6dT is spaced from the oligomer by a linker arm with a total of 10 atoms.

### 3.1. Synthesis, purification and analysis of oligonucleotide–amino acid/peptide conjugates

**3.1.1. Synthesis of oligonucleotide–amino acid/peptide conjugates.** After optimizing all experimental conditions, a common procedure was adopted for synthesis of oligonucleotide conjugates of amino acids or peptides.[10] In a typical method; 300 μl of EDC (0.1 M) in DMF was added to 100 μl acetyl amino acid (0.1 M) in DMF solution and stirred well for 10 min. This was followed by the addition of 100 μl oligomer (1–2 mg/ml) and 300 μl buffer at pH 7.0 (potassium phosphate 0.1 M). The reaction mixture was stirred well and kept overnight. Formation of the conjugate product was analyzed by anion exchange HPLC. The yield was calculated by manual integration and from the ratio of area of product peak to reactant peak. The solution of EDC was prepared freshly every time although it was reported that EDC solution is quite stable for 5 h at 25°C and neutral and higher pH regions.[21]

**3.1.2. Purification of conjugates.** The synthesized oligonucleotide–amino acid/peptide conjugates were purified using a C18 Sep-Pak reverse phase cartridge from Waters. For this purpose, the reaction mixture was initially vacuum dried to evaporate all solvents and resuspended in 1 ml of milli Q water. A Sep-Pak cartridge was prepared by passing 10 ml HPLC grade acetonitrile followed by 10 ml of milli Q water prior to loading of oligomer conjugate with a 1 ml disposable of syringe. The filtrate was reloaded 2–3 times to ensure complete loading of the sample. Next, the cartridge was washed thoroughly with milli Q water. It was observed that extra washings were needed for acetyl-tryptophan or acetyl-tyrosine conjugates. The oligonucleotide conjugate

was then eluted using 40% of acetonitrile; collected fractions were collected together and vacuum dried.

**3.1.3. Analysis of conjugates.** The progress of the conjugation reaction and analysis of conjugation product was followed by HPLC using an analytical anion exchange column (Waters Gen Pak FAX, 4.6×100 $mm^2$) and employing a Waters 996 liquid chromatography system consisting of a Waters 600 multi solvent delivery system with photodiode array detector. In a typical solvent system, buffer A=0.1 M sodium acetate in 10% acetonitrile at pH 6.5, and buffer B=40 mM sodium acetate and 1 M NaCl in 10% acetonitrile at pH 6.5 was used. A linear gradient of 0–40% B in 50 min, with flow rate of 0.75 ml/min was used for elution of conjugates. Due to interference of DMF and the acetyl amino acid in this system, each conjugate product was purified initially on C18 Sep-Pak cartridge and was concentrated prior to HPLC analysis. Unless and otherwise stated, an aliquot of reaction mixture was usually withdrawn for HPLC analysis after 24 h. Millennium 2010 software was used for analysis of the HPLC results.

**3.1.4. Mass spectrometry.** All mass spectra for oligomers, peptide or oligomer conjugates were obtained using a linear time of flight, Finnigan Mat Vision 2000 mass matrix assisted laser desorption and ionization spectrometer (MALDI). The system was operated in positive mode for small oligomers like GGT*GG and in negative mode for higher mass oligomers. For laser desorption and ionization, a nitrogen laser with wavelength of 337 nm was used. The vacuum inside the 1.7 m flight tube was always kept between $10^{-7}$ and $10^{-6}$ Torr and a high accelerating voltage kept between 28 and 31 kV.

**3.1.5. Sample preparation of MALDI-MS.** The conjugation products purified on C18 Sep-Pak cartridge were vacuum dried and redissolved in 20–50 μl milli Q water to a concentration about 150–200 μg/ml. 3-Hydroxypicolinic acid (HPA) purchased from Aldrich was used as matrix without further purification. 40–50 mg/ml solution of HPA in water was prepared freshly in 1:1 water–acetonitrile system and kept on ice for few minutes before loading the sample. Sample (1–2 μl) and matrix (8–9 μl) were thoroughly mixed prior to analysis with a typical working molar ratio of matrix to analyte between 2000:1 and 10000:1. For MS analysis, 1 μl of each matrix–sample mixture was loaded at least as 3–5 spots with different sample matrix ratios on a stainless steel grid plate. Sample spots were dried under forced air current at room temperature. The plate was then inserted into the mass spectrometer. A good spectrum was usually obtained when the sample spot was composed of oblong transparent crystals of HPA matrix with the sample being located at the edges of circle. Calibration for accurate mass measurement was obtained by adding a known amount of internal standard. Even though a single spectrum produced in one laser shot was good enough to get accurate mass of the sample, typical spectra produced from 20–60 laser shots were averaged in order to improve the signal to noise ratio.

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