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A novel method of introducing hydrophobic moieties into oligonucleotides for covalent and non-covalent immobilization on electrode surfaces

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Abstract

An effective method for the introduction of oleylamine-modified cytidine units into predetermined position(s) of the oligodeoxyribonucleotide (ON) chain during automated ON synthesis has been developed. The high yields of the condensation products upon the introduction of the modified units allow the methods suggested to be used for the synthesis of ONs with two hydrophobic substituents. We also suggest a simple method for obtaining ONs with 5′ -terminal hydrophobic linker with free thiol group. The functionality of synthesized ON modified by thiol group and that with hydrophobic spacer for the detection DNA hybridization has been approved in conductometric experiments. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Oligodeoxyribonucleotide; DNA sensors; Hybridization; Conductivity

1. Introduction

In the last few years, the field of nucleic acids chemistry dealing with their electrochemical properties has intensively developed [1,2]. This is due to the possibility of using electrodes with immobilized ONs for the detection of nucleic acids of pathogenic microorganisms. The development of effective methods for the immobilization of nucleic acids on electrode surfaces is therefore an important problem.

The two possible ways for the immobilization of DNA fragments are noncovalent and covalent binding with electrode surfaces. The first variant provides for the use of moieties of hydrophobic molecules, such as lipids and fatty acids, introduced into ON chains and capable to bind with hydrophobic electrode surfaces (e.g., graphite paste or lipid membranes) [1,2]. In the second case, the introduction of reactive groups which are capable to react with a surface of electrodes is required. The methods for the introduction of the hydrophobic molecules to ON termini, both during automated solid-phase synthesis [3–5] and postsynthetically [6,7], were reported. However, the methods suggested are not versatile and cannot be used for simultaneous modification of 3′ - and 5′ -terminal fragments nor for the introduction of intrachain modified units.

Here, we propose a method for the introduction of hydrophobic moieties for non-covalent immobilization that involves the introduction of the modified unit into any predetermined position of the ON chain during automated phosphoramidite synthesis. We also suggest a method for the introduction of hydrophobic linkers carrying terminal mercapto groups for covalent immobilization of ONs onto gold electrodes covered by lipids. The ON terminated with mercapto groups has been used for the fabrication DNA sensor. The hybridization of DNA has been tested by conductometry.

2. Experimental

2.1. Chemical modification of ONs

TLC was performed on the Silica gel 60 $_{254}$F (Merck, Germany) precoated plates in (A) 95:5 chloroform–ethanol,

Abbreviations: CPG, controlled pore glass; TPSCI, 2,4,6-trisopropylphenylsulfonyl chloride; ON, oligodeoxyribonucleotide; MUA, 11-mercaptoundecanoic acid.

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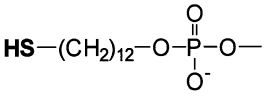
Table 1

Oligonucleotides	Primary structure 5′ → 3′	Mass-spectra MALDI, *m/z* calc./exp.
(I)	CACCTTGCTGAAATTTTCCC *	6267.4/6266.0
(II)	CAC * CTTGCTGAAATTTTCCC *	6531.9/6529.6
(III)	C * ACCTTGCTGAAATTTTCCC *	6531.9/6526.7
(IV)	CAC * CTTGCTGAAATTTTCCC	6267.4/6265.8
(V)	C * ACCTTGCTGAAATTTTC	6267.4/6268.4
(VI)	AC * CTTGCTGAAATTTTCCC *	
(VII)	ACCTTGCTGAAATTTTCCC *	
(VIII)	AC * CTTGCTGAAATTTTCCC	
(IX)	XTTTTTTTTTT	3260.9; 6521.8** /3262.4; 6524.8 * *
(X)	XCCCAAAAGTGAAAACAC	5445.4/5446.9
(XI)	XACCTTGCTGAAATTTTCCC	
(XII)	XCCTTCAATATGACTTTTATC	

C * = 2′ -deoxy-5-methyl-N_4-oleyl.

X= 

M* * = product of –S–S-dimerisation.

(B) 9:1 chloroform–ethanol, and (C) 98:2 methylene chloride–triethylamine systems. Column chromatography was carried out on Silica gel 60 (Merck, Germany) in an ethanol gradient in chloroform [(D), 0–5% and (E), 0–15%].

Temperature-dependencies of UV absorption at 260 nm and UV spectra were registered on a Hitachi 150-20 (Japan) spectrophotometer supplied with a thermostated cell holder in a 1-cm quartz cell, mass spectra were measured on a Finnigan MAT VISION 2000 TOF (MALDI) mass spectrometer.

5′ -*O*-Dimethoxytrityl-3′ -*O*-trimethylsilylthymidine was prepared from **(1)** as described in Ref. [8] and used without isolation for the synthesis of **(2)**.

1-(5′ -*O*-Dimethoxytrityl-2′ -deoxy-β-D-ribofuranosyl)-4-(1-triazolyl)-5-methyl-2-pyrimidone **(2)**. Triazole (6.4 g, 93 mmol) was suspended in acetonitrile (50 ml) and phosphorus (V) oxychloride (1.96 ml, 16.9 mmol) was added. Then triethylamine (15 ml, 110 mmol) was added dropwise at stirring and cooling on ice, the reaction mixture was kept for 30 min at 20 °C, and a solution of 5′ -*O*-dimethoxytrityl-3′ -*O*-trimethylsilylthymidine (5 mmol) in acetonitrile (20 ml) was added. The reaction was performed for 15 min at 20 °C (TLC in system A). The reaction mixture was washed with water, the organic layer was concentrated in vacuo and chromatographed on a Silica gel column in system E to yield 1.42 g (48%) of **(2)**; R_f 0.75 (A).

5′ -*O*-Dimethoxytrityl-2′ -deoxy-5-methyl-N^4-oleylcytidine **(3)**. Compound **(2)** (0.3 g, 0.5 mmol) was dissolved in dioxane (5 ml), and oleylamine (1.5 ml, 4.6 mmol) was added. The conversion at 20°C was monitored by TLC (system B). After 1 h, the reaction mixture was concentrated in vacuo and chromatographed on a Silica gel column in system D to give 0.36 g (90%) of **(3)**; R_f 0.7 (B).

5′ -*O*-Dimethoxytrityl-2′ -deoxy-5-methyl-N^4-oleylcytidine 3′ -(*N*,*N*-diisopropylamido)-β-cyanoethylphosphite **(4)** was obtained from **(3)** according to [9].

Immobilization of 5′ -*O*-dimethoxytrityl-2′ -deoxy-5-methyl-N^4-oleylcytidine **(3)** on the polymeric support CPG-500 was performed by the method reported in Ref. [10].

12-(*S*-trityl)mercaptododecanol-1 **(6)**. Solution of NaOH (0.48 g, 12 mmol) in 4.5 ml of water was added to 30 ml of ethanol/benzene (1:1) solution of tritylmercaptane (2.76 g, 10 mmol). After the precipitate formed was dissolved by stirring, the solution of 12-bromdodecanol-1 (1.326 g, 5 mmol) in 20 ml of ethanol/benzene (1:1) was added. The reaction was allowed for 8 h, filtered and dried in vacuo. The residue was dissolved in chloroform and washed with 5% aqueous $NaHCO_3$ (2 × 25 ml), saturated NaCl (50 ml), dried, concentrated in vacuo and chromatographed on Silica gel column in system D to yield 2.2 g (95%) of **(6)**, R_f 0.25 in chloroform.

MALDI MS **(6)**: calc. $C_{31}H_{40}OS$ M = 460.7, found *m/z* 459.7.

N,*N*-Diisopropylamido-(β-cyanoethyl)-(12-tritylmercaptododecyl)phosphite **(7)** was obtained from **(6)** analogously to the method described in Ref. [9].

Synthesis of ONs (**I–VIII** Table 1) with oleyl-containing units was carried out on an Applied Biosystems 380B automated DNA synthesizer (USA) by the phosphoramidite method using 0.12 M solutions of modified phosphoramidites and the condensation time increased up to 4 min, with the resulting ONs deprotected as described in Ref. [9]. ONs with hydrophobic residues were isolated by reverse-phase HPLC on a Gilson instrument (USA) using a Beckman Ultrasphere Octyl (4.6 x 250 mm) column and a 0–80% linear gradient of acetonitrile in 20 mM ammonium acetate as a mobile phase (80 min).

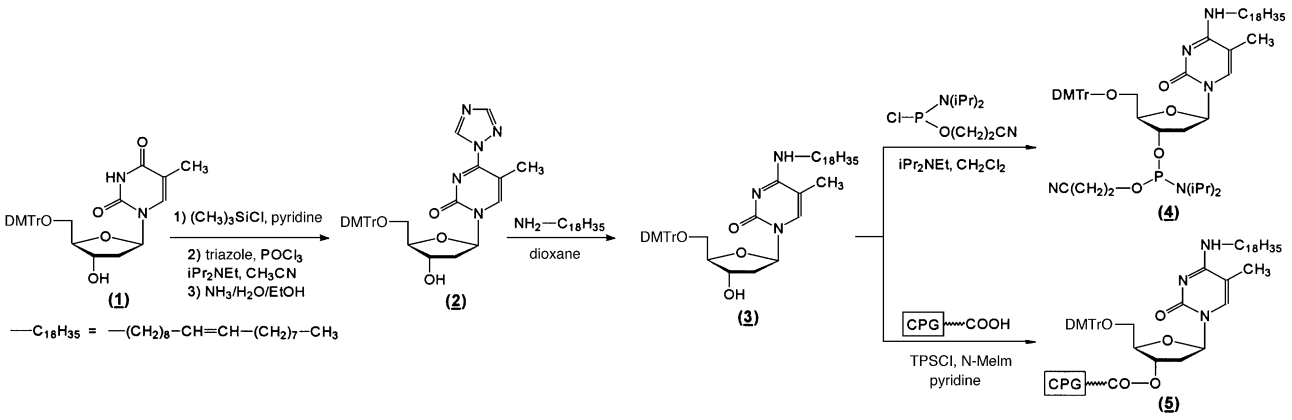


Fig. 1. Synthesis of monomeric synthons for the incorporation of oleyl moieties into ONs.

ONs (**IX–XII**, Table 1) containing 5′ -mercaptododecyl linker were synthesised as described above. 12-Tritylmercaptododecylphosphite **(7)** was coupled to 5′ -termini of ONs during the last stage of the synthesis. Trityl protecting group was removed from sulfur after ON cleavage from polymer support by subsequent treatment with aqueous $AgNO_3$ (40 min) and decomposition of forming salt by aqueous DTT (5 min). ONs obtained were isolated by reverse-phase HPLC.

ON (**XIII**) (5′ -ACCTTGCTGAATTTTCCC-$(PO_2)^-$-SH) containing 3′ -phosphorothioate group was synthesized as we described previously in Ref. [11].

Ligation with T4 DNA Ligase was carried out at 4 °C overnight, with equimolar amounts of ONs and threefold excess of matrix. The products were identified by relative mobility in PAGE (20%).

2.2. Preparation of DNA sensor and conductometric detection of DNA hybridization

The DNA sensor has been prepared as described elsewhere [11] by means of chemisorption of thiolated oligonucleotides onto gold support sputtered on a smooth silicon plate (diameter 0.5 mm). The clean gold support has been first immersed into 1 μM water solution of oligonucleotides (without and/or with hydrocarbon spacer) for 10 min and then into 1 mM ethanol solution of MUA for 1 h. The MUA forms an insulating layer on a gold support, i.e. covers the free gold surface. For the fabrication of the sensor we used thiolated ONs described in this work.

The measurement of the current flow across the supported thin films was performed with a programmable electrometer, Keithley 6512 (USA), connected on-line with an IBM PC 486 DX through KPC-488.2AT Hi Speed IEEE-Interface board [11]. In all experiments, the following buffer has been used: 0.1 M NaF + 10 mM Tris–HCl + 2 mM EDTA (Sigma), pH 7.6. pH has been adjusted by the addition of a small amount of 0.1 M NaOH. NaF was used in order to avoid the interference of chlorine anions with gold support. The experiments were performed at T=(20 ± 1 °C).

3. Results and discussion

3.1. Chemical modification of ONs

To obtain ONs with oleylamine residues, we employed the method involving the reactive thymidine derivative bearing a triazole residue in C4-position. The 5′ -*O*-protected thymidine **(1)** was modified by the standard procedure [12]. 1-(5′ -*O*-Dimethoxytrityl-2′ -deoxy-β-D-ribofuranosyl)-4-(1-triazolyl)-5-methyl-2-pyrimidone **(2)** was reacted with oleylamine to give 5′ -*O*-dimethoxytrityl-2′ -deoxy-5-methyl-N^4-oleylcytidine **(3)**. Compound **(3)** was treated with ***N***,*N*-diisopropylamido-β-cyanoethylchlorophosphite to obtain the monomeric synthon **(4)** for ON synthesis (Fig. 1).

Phosphoramidite derivative **(4)** was involved into standard automated ON with no less than 95% yield. In order to obtain ONs with a modified unit at the 3′ -end, nucleoside **(3)** was immobilized on the CPG-500 polymeric support according to the scheme presented in Fig. 1 to yield **(5)**. Using compound **(4)** and/or polymeric support **(5)**, ONs (**I–VIII**, Table 1) with oleylamine moieties were synthesized with sequences complementary to the DNA fragments of the pathogenic bacterium *Salmonella*.

In connection with the data obtained for thermodynamic stabilities of duplexes formed by ONs bearing hydrophobic substituents, we should expect ONs modified at the chain ends to hybridize effectively with complementary DNA

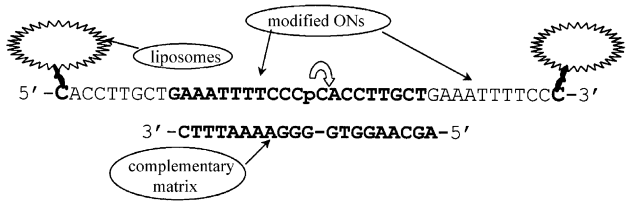


Fig. 2. Incorporation of oleyl-containing ONs into the surface of liposomes.

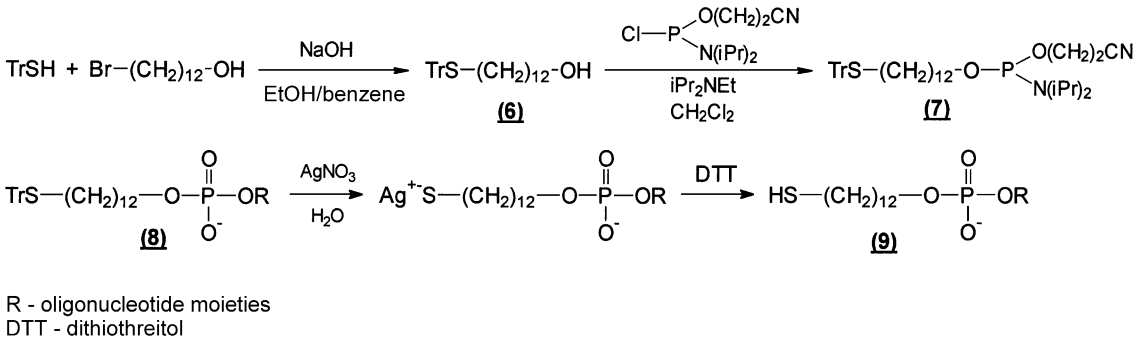


Fig. 3. Synthesis of ONs containing thiol linkers at 5′ -termini.

sequences from solution upon the immobilization on the hydrophobic electrode surface.

ONs containing oleylamine moieties introduced into the surface of liposomes could easily form duplexes in the presence of complementary DNA in solution. This fact was confirmed by the enzymatic ligation of two ONs containing 5′ - and 3′ -terminal oleyl moieties in the system mentioned above, as it is shown in Fig. 2.

The data obtained show that the presence of liposomes has no negative influence on the efficiency of complementary interactions between ONs containing oleyl residues. It was confirmed by the fact that yields of ligation products obtained in the presence of liposomes (65%) did not differ from those obtained in aqueous solutions (65–70%).

It is convenient to use ONs containing terminal hydrophobic "tails" with thiol groups for the immobilization on golden electrode surfaces covered with hydrophobic layer. We have elaborated an original scheme of introducing 12-thiododecanol-1 at 5′ -termini of ONs in the process of automated solid phase phosphoramidite DNA synthesis without changing the standard protocols. We have chosen a standard approach to synthesize ONs with 12-mercaptododecyl groups attached to 5′ -terminal phosphate: utilizing a compound which will introduce desirable 5′ -terminal modification, *N*,*N*-diisopropylamido-(β-cyanoethyl)-(12-tritylmercaptododecyl)phosphite **(7)**, in the final coupling step. Synthesis of **(7)** included two steps: nucleophilic replacement of bromine in 12-bromododecanol-1 and phosphitylation of hydroxyl group in the resulting compound **(6)**. It was conducted according to the scheme presented in Fig. 3.

A set of ONs (**IX–XII**, Table 1) containing 5′ -terminal TrS-group M**(8)** has been synthesized using phosphoramidite derivative **(7)** with sequences complementary to the fragments of pathogenic bacteria *Salmonella* genome. The final synthetic step was the selective removing of trityl group from ONs **(8)** isolated by HPLC with silver nitrate and the subsequent reduction with dithiothreitol to give free terminal SH-group in ONs **(9)**.

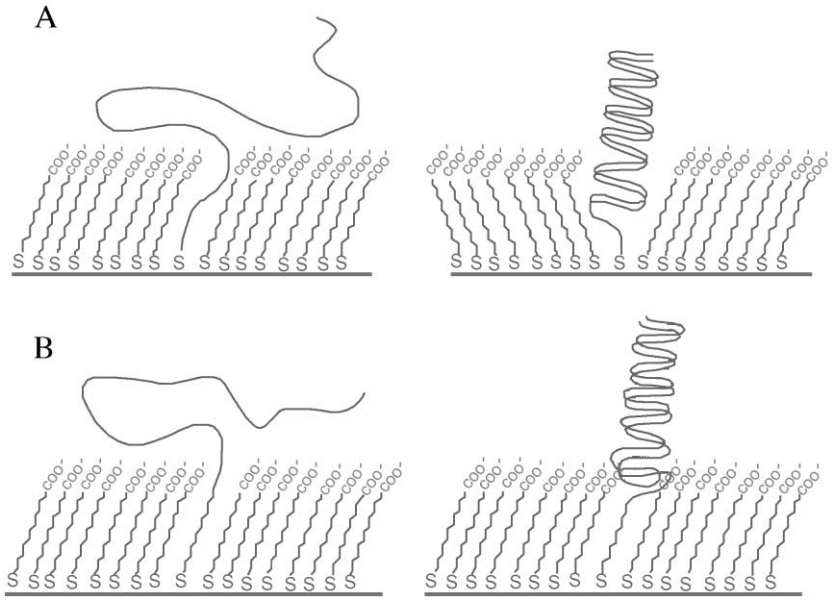


Fig. 4. Schematic representation of the DNA sensors formed by single-stranded DNA without (A) and with (B) spacer at the surface of gold layer covered by amphiphilic layer of MUA. Right side: hybridization with complementary ON.

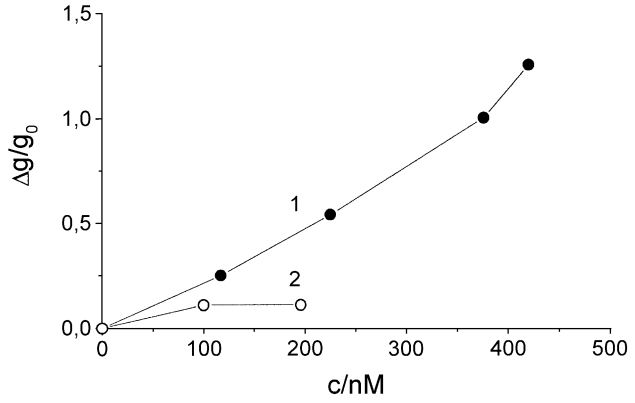


Fig. 5. Changes of conductivity of the DNA sensor as a function of concentration of complementary strand for sensors formed by single-stranded DNA without (1) and with (2) hydrophobic spacer. $\Delta g = g - g_0$, where g is the conductivity of the sensor at a certain concentration of complementary strand and g_0 is the conductivity of the sensor in the absence of complementary chain.

3.2. Detection of DNA hybridization

The principal difference between DNA sensors based on single-stranded DNA without and with hydrocarbon spacer (12 methylene groups) (ONs **XIII** and **XI**, respectively) is obvious from Fig. 4, where these two types of sensors are schematically presented. It can be seen that while for the sensor without spacer (immobilized ON **XIII**), certain distortion of the amphiphilic layer could take place following the hybridization with complementary strand, for the sensor with spacer (immobilized ON **XI**), the changes in the ordering of the layer should not be expected. These peculiarities are crucial for the detection of hybridization by means of measuring the conductivity of the layers. The relative changes in the conductivity for these sensors as a function of concentration of complementary chain are presented in Fig. 5. We can see that while for the sensor without spacer the conductivity increases, for the second type of sensor the changes in conductivity are less expressed. Moreover, at a higher concentration of complementary strand ($c > 300$ nM) we observed the decrease in conductivity. Thus, for the conductometric detection method the DNA sensor without spacer would be preferable, while, e.g., for mass detection, the sensor with spacer could be more advantageous due to better access of complementary strand to the sensor surface. The possible mechanisms of the changes in conductivity of the DNA sensor have been discussed earlier [11]. We assume that the insulating properties of the amphiphilic layer decrease due to the hybridization process followed by distortion.

4. Conclusion

Thus, we have developed an effective scheme for the introduction of oleylamine-modified cytidine units into predetermined position(s) of the ON chain during automated ON synthesis. The high yields of the condensation products upon the introduction of the modified units allow the methods suggested to be used for the synthesis of ONs with two hydrophobic substituents. We also suggest a simple method for obtaining ONs with 5′-terminal hydrophobic linker with free thiol group. The functionality of synthesized ON containing thiol groups and that with hydrophobic spacer for the detection of DNA hybridization have been proved by conductometric measurements.

Acknowledgements

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