Oligonucleotide Synthesis on Maleic Anhydride Copolymers Covalently Bound to Silica Spherical Support and Characterization of the Obtained Conjugates

CAROLE CHAIX, CLAIRE MINARD-BASQUIN, THIERRY DELAIR, CHRISTIAN PICHOT, BERNARD MANDRAND

Laboratoire de Chimie et Biochimie Macromoléculaire, UMR 103-bioMérieux, Ecole Normale Supérieure de Lyon, 46 Allée d'Italie, F-69364 Lyon, France

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ABSTRACT: A new route was proposed to make polymer-oligonucleotide conjugates of potential applications in diagnostics. It consisted in direct synthesis of oligonucleotides onto controlled pore glass surface grafted with poly(maleic anhydride-*alt*-methyl vinyl ether) (P[MAMVE]) or poly(maleic anhydride-*alt*-ethylene) (P[MAE]). The anhydride moieties were used for both the covalent coupling of the copolymer *via* ester bond and binding of 5′-dimethoxytrityl thymidine 3′-(6-aminohexyl phosphate) (**I**) initiator of oligodeoxynucleotide (ODN) synthesis *via* amide bond. The difference of stability between ester and amide links under basic treatment was used for the selective cleavage of (polymer-oligonucleotide) conjugates after DNA synthesis completion. We succeeded in grafting functionalized copolymer onto silica surface and Polythymidine 26-mer ODN was performed. After concentrated ammonium hydroxide treatment, conjugate crude materials were characterized by size exclusion chromatography coupled to multiangle laser light scattering detection. The number average molecular weight ($\overline{Mn}$) for conjugate with P[MAMVE] was abnormally lower than expected and was assigned to polymer degradation using high pH conditions. Such a phenomenon did not occur with P[MAE]-polythymidine conjugate. However, in both cases, parasite ODN synthesis was also evidenced, which was attributed to thymidine phosphoramidite adsorption side reaction during DNA synthesis. J Appl Polym Sci 70: 2487–2497, 1998

Key words: maleic anhydride copolymer; oligodeoxynucleotide; solid-phase synthesis; light scattering size exclusion chromatography; silica support; conjugate; adsorption

INTRODUCTION

In the field of infectious diseases, diagnostic tests aim at quantifying the presence of the pathogen by analyzing body fluids, such as blood or serum (i.e., whole blood minus the cells), urine, Cerebro Spinal Fluid (CSF), etc.). A particular antigen is present in the patients' sera at minute concentrations, whereas sera contain a total amount of 80 gL^{-1} of proteins. Thus, there is a paramount need of high specificity and sensitivity to perform efficient diagnostic tests. One way to achieve efficient testing is to fish the antigen out of the body fluid using "biospecific solid supports" for easier quantification in a second step. These solid supports bear a biological molecule capable of recognizing a specific antigen. On incubation of the sample, the specific analyte, if present, will strongly bind to the support, whereas other proteins will be loosely immobilized. A washing step will remove the poorly bound nonspecific molecules from solid support. Therefore, this method performs a spe-

Correspondence to: C. Chaix.

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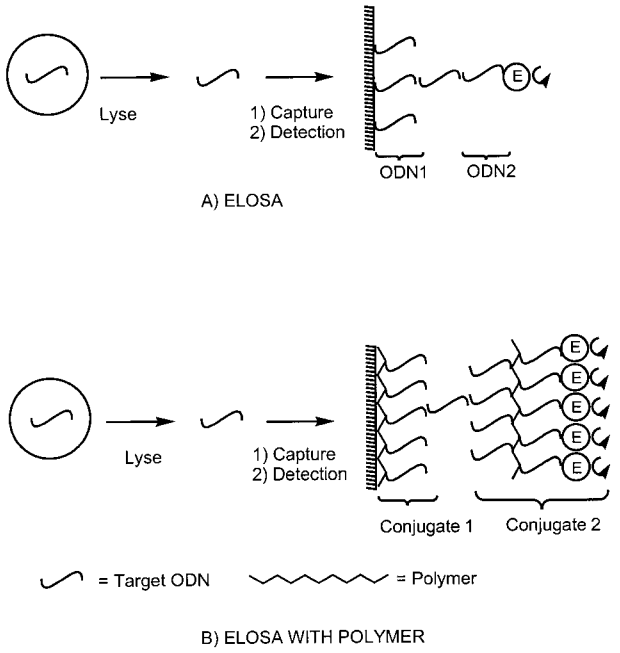


Figure 1 Principle of diagnostic test ELOSA and ELOSA with polymer. E = enzyme; ODN1 = capture sequence; ODN2 = detection sequence; conjugate 1 = [polymer-ODN1]; conjugate 2 = [polymer-ODN2].

cific isolation of the analyte of interest from the very complex body fluid to allow accurate quantification of the presence of the antigen in the sample. In immunology, this technique is widely used and known as ELISA (Enzyme Linked Immunosorbent Assay).[1] Recently adapted from the immunoassay field, a closely related method was applied for genetic diagnostics and called ELOSA (Enzyme Linked Oligosorbent Assay).[2] In genetic diagnostics, the presence in a sample of viruses or bacteria is detected by looking for their genomes. This new test is described in Figure 1(A) and is comprised of a solid support on which a specific oligonucleotide is immobilized. After incubation of the sample, if the target DNA is present, it will specifically hybridize to the capture probe whereas non-specific material will be loosely bound and removed at the washing step. Detection is performed by incubation of a probe bound to an enzyme. This probe hybridizes to the target DNA, and, after washing off the loosely bound excess reagent, the enzymatic substrate is introduced and the enzymatic reaction produces a fluorescent signal, for instance, the intensity of which is directly related to the amount of target DNA bound on the biosurface. To increase the sensitivity of the tests, instead of using mere oligonucleotides on the solid phase, polymers of oligodeoxynucleotides (ODNs) can be used to increase the loading of the solid support and so improve the capture efficiency of the target. Likewise, for the detection step, polymers of the detection probe can be used; thus, concentration of the enzyme is increased, which results in an increase in detection signal [Fig. 1(B)]. Both of these concepts were described in patents originating from this lab[3,4] and were successfully used for the detection of the hepatitis B virus DNA.[5] Various polymers have been tested, and all of them were based on the use of a reactive comonomer capable of binding amino counterparts by the formation of amide or imine bonds. Hence, linear synthetic functional *N*-vinyl pyrolidone-*N*-acryloxy succinimide,[4–7] maleic anhydride copolymers,[8,9] and glucose amine polymers[10] have been described to covalently bind prepurified oligonucleotides. One of the shortcomings of this approach was the formation of polymer/ODN aggregates limiting the accessibility for the oligonucleotides entrapped within the aggregates.[8,11] Furthermore, this strategy of immobilizing ODNs onto reactive polymers does not allow us to envision simultaneous immobilization of biomolecules of a different nature (i.e., ODNs and peptides) onto polymer chains whose respective coupling conditions are quite different.

In this context, we have focused on another alternative consisting of synthesizing biomolecules, such as oligonucleotides or peptides, directly from a linear organic polymer grafted onto a solid support. Then, at the end of synthesis, the corresponding polymer-biomolecule conjugate can be selectively cleaved from the support to be released in solution.

In the literature, both silica gel beads[12,13] and biochips[14] have been coated by an amino-modified polypropylene film to initiate oligonucleotide synthesis. The presence of polymeric film at the interface between solid surface and nucleic acid optimizes access to all dissolved reagents, leading to syntheses of high quality. In addition, the film exhibits a high loading capacity. However, no mention of postsynthesis liberation in solution of the polymer-ODN conjugate was pointed out.

In this article, we report on preliminary investigations on the concept of solid-phase syntheses of polymer-ODN conjugates initiated from new controlled pore glass (CPG) supports, surface-grafted with linear poly(maleic anhydride-*alt*-methyl vinyl ether) (P[MAMVE]), or poly(maleic anhydride-*alt*-ethylene) (P[MAE]). Reactive an-

hydride functions of the polymers were used for both tethering the macromolecule at the surface of the solid support and for coupling biomonomers that will initiate DNA synthesis.

MATERIALS AND METHODS

All solvents were analytical or HPLC grade. General reagents were purchased from Aldrich (Milwaukee, WI) and Sigma Chemical Co. (St. Louis, MO). Chromic-sulfuric acid solution was from Prolabo (Lyon, FR). CPG (mean pore diameter of 2000 Å; 40–85 μm particle size; 9.2 $m^2\ g^{-1}$ of surface area) was ordered from Fluka (Ronkonkoma, NY). Glass beads (100–150 μm) were furnished by Polysciences, Inc. (Warrington, PA). Infrared (IR) analyses were done on a Nicolet FITR 5PC, in the diffuse reflectance mode [Diffuse Reflectance Infrared Fourier Transform (DRIFT) spectroscopy]. ^{1}H-NMR spectra were recorded on AM400 Brüker spectrometer operating in the Fourier transform mode. The ^{1}H chemical shifts were expressed in ppm, with reference to trimethylsilane. Assignment of ^{1}H signals was achieved by two-dimensional ^{1}H-^{1}H homonuclear correlated NMR experiments (COSYGS). Fast-atom bombardment (FAB) mass spectrometry analyses were conducted on a model VG micromass ZAB 2-SEQ spectrometer in a positive mode detection on a thioglycerol matrix. P[MAMVE] was an alternated copolymer of maleic anhydride and methyl vinyl ether supplied by Polysciences, Inc. [average number molecular mass ($\overline{Mn}$): 67,000 g mol^{-1}]. P[MAE] was an alternated copolymer of maleic anhydride and ethylene supplied by Polysciences, Inc. [average number molecular mass ($\overline{Mn}$): 27,000 g mol^{-1}]. Oligodeoxyribonucleotide syntheses were performed on an expedite nucleic acid synthesis system instrument (Millipore, Corp., Bedford, MA) using standard DNA cyanoethyl *N,N*-diisopropylamino phosphoramidite chemistry. Capillary zone electrophoresis was done on an Applied Biosystems instrument equipped with a silica capillary (72 cm × 50 μm). Size exclusion chromatography (SEC)-multiangle laser light scattering (MALLS) experiments were performed on-line with the following size exclusion high-performance chromatography set-up. Two associated columns (Waters Ultra-Hydrogel 500 and 1000 or Waters Ultra-Hydrogel 1000 and 2000) and a Waters 510 high-performance liquid chromatography pump were running in a boric acid buffer (pH 10) as eluent and at a flow rate of 0.5 mL min^{-1}. For the detection part, a Waters 484 absorbance detector, a Waters 410 differential refractometer, and a three-angle MINI DAWN F detector (Wyatt Technology Santa Barbara, CA) operating at 690 nm were used on-line.

Synthesis of 5′-Dimethoxytrityl-2′-deoxythymidine-3′-(6-aminohexyl phosphate) (I)

Two hundred milligrams (0.37 mmol) of 5′-dimethoxytrityl-2′-deoxythymidine were dried *via* anhydrous pyridine, and anhydrous acetonitrile coevaporations then dissolved in 7.5 mL of acetonitrile. 1H-Tetrazole (0.55 mmol of a 0.35*M* solution/acetonitrile) and 6-(trifluoroacetylamino)hexyl-(2-cyanoethyl)-(*N,N*-diisopropyl)-phosphoramidite (0.44 mmol of a 0.3*M* solution/acetonitrile) were slowly added through the septum, and the solution was stirred at room temperature. After 1 h, 4 mL of iodine solution [100 m*M* iodine in 2% H_2O, 20% pyridine, and 75% tetrahydrofuran (THF)] were added dropwise in 5 min, and the mixture was stirred for an additional 10 min before being concentrated under reduced pressure. The residue was dissolved in CH_2Cl_2, and the organic mixture was washed once with 5% $NaHCO_3$, twice with H_2O, dried over Na_2SO_4, and evaporated to dryness *in vacuo.* Then, the residue was resuspended in a solution of 700 μL of ethanol and 6 mL of NH_4OH (30%, aqueous), and the deprotection reaction was achieved in 16 h at 60°C in a hermetical flask. After concentration *in vacuo,* the residue was chromatographed on a column of silica gel with (CH_2Cl_2/triethylamine 95 : 5) and methanol gradient to give monomer **I** (123 mg, 46% yield). ^{1}H-NMR [dimethylsulfoxide (DMSO)-*d*6]: 1.32 (m, 2H, C—CH_2—C), 1.37–1.42 (m, 4H, PO—CH_2—$\underline{CH_2}$—$\underline{CH_2}$—C), 1.39 (s, 3H, CH_3), 1.55 (t, 2H, C—$\underline{CH_2}$—CH_2—NH_2), 2.21–2.34 (m, 2H, H_2', H_2''), 2.71 (t, 2H, C—$\underline{CH_2}$—NH_2), 3.19 (m, 2H, H_5', H_5''), 3.57 (t, 2H, PO—CH_2—C), 3.71 (s, 6H, O—CH_3), 4.07 (s, 1H, H_4'), 4.65 (s, 1H, H_3'), 6.16 (t, 1H, H_1'), 7.21–7.36 (m, 13H, H Ar), 7.46 (s, 1H, H6). FAB MS: *m/z* 724, [M + H^+]; FAB High Resolution Mass Spectrometry (HRMS): *m/z* 724.3009 (Calcd for [M + H^+] 724.299).

CPG or Glass Bead Functionalization

The functionalization step was adapted from a previous procedure published by Southern and

colleagues.[15] Three grams of underivatized CPG or 5 g of glass bead were suspended in 6 mL of a chromic-sulfuric acid solution [saturated solution of chrome (VI) oxide in 95% sulfuric acid]. After 3 h of activation at 110°C, a majority of silane on surface was under silanol groups. Supports were filtrated and carefully washed with water. After a rapid wash with dry acetone, supports were dried *in vacuo* for half an hour and immediately used for silanization.

Activated beads were suspended in 28 mL of a solution (6.1 mL of 3-glycidoxypropyltrimethoxysilane, 1.7 mL of triethylamine, and 20.2 mL of dry toluene). Supports were gently stirred overnight at 90°C, then washed thoroughly with anhydrous acetone and dried for 3 h at 110°C.

In a second step, these beads were suspended in 10 mL of hexaethylene glycol (HEG) with 6 μL of sulfuric acid (>95%). The reaction was gently stirred overnight at 90°C, then the beads were washed with anhydrous acetone and dried in desiccator.

In addition to reaction with diol, 100 mg of epoxy CPG were hydrolyzed for 3 h in 20 mL of (0.2*N* HCl) solution to cleave the epoxide residue to yield a primary hydroxyl group. Then, CPG was washed with water and anhydrous acetone before drying in desiccator.

Quantification of Accessible Hydroxyl Groups on the Surface through Tritylation Reaction

Fifty milligrams of functionalized CPG and 100 mg of functionalized glass beads were dried by anhydrous pyridine coevaporation in separate flasks and resuspended in 1 mL of pyridine. Then, 10 mg (29.5 μmol) and 20 mg (59 μmol) of 4,4′-dimethoxytrityl chloride were added, respectively. The reaction was stirred under argon for 20 h at room temperature and then quenched with methanol. After filtration, beads were successively washed with 5% $NaHCO_3$, water, and acetone and dried *in vacuo* for several hours. Acidic treatment with 3% trichloroacetic acid in CH_2Cl_2 and measurement of optical density at λ = 498 nm (Σ_{Dmt} = 70 mL μmol^{-1} cm^{-1}) gave an estimate hydroxyl concentration on the surface of 16.2 μmol g^{-1} for CPG-HEG (1.76 μmol m^{-2}), 14.6 μmol g^{-1} for CPG-HCl, and 0.138 μmol g^{-1} for glass beads (7.27 μmol m^{-2}).

General Procedure for Grafting of Conjugate (P[MAMVE]-Nucleotide (I)) to Beads

Fifteen milligrams (224 nmol) of polymer were dissolved in 1 mL of anhydrous DMSO at 37°C. In parallel, 10 mg (13.8 μmol) of (**I**) were dissolved in 1 mL of the same solvent. To an appropriate volume of anhydrous DMSO permitting a final volume of 500 μL for reaction were successively added 2.2 nmol of polymer (0.96 μmol of anhydride function), 0.2 μmol of nucleotide (**I**), and 0.1 μmol of 4-dimethylaminopyridine (DMAP) (see Results and Discussion section). After stirring at room temperature for 1 h, 90 mg of silica beads were added to the mixture, and the grafting reaction was allowed to proceed overnight. Supports were then filtrated and carefully washed with DMSO and anhydrous acetone before being dried *in vacuo* for several hours. Dimethoxytrityl amount on the surface was estimated by acidic treatment of silica as previously described.

General Procedure for Grafting of Conjugate (P[MAE]-Nucleotide (I)) to Beads

P[MAE] was first dried 24 h at 120°C in an oven. 12.1 mg (448 nmol) of polymer were dissolved in 1 mL of anhydrous DMSO at 37°C. In parallel, 10 mg (13.8 μmol) of **I** were dissolved in 1 mL of the same solvent. To an appropriate volume of anhydrous DMSO, permitting a final volume of 500 μL for reaction, 13 nmol of polymer (2.88 μmol of anhydride function), 0.6 μmol of nucleotide **I,** and 0.3 μmol of DMAP were successively added. After 4 h of coupling reaction at room temperature, 90 mg of silica beads were added and the reaction proceeded as previously described.

ODN Synthesis

Functionalized support (0.1 μmol) was used for syntheses with a standard 0.2 μmol coupling cycle procedure. Before syntheses, a capping step consisting of 3 runs of the capping cycle (acetic anhydride/lutidine/THF; *N*-methylimidazole/THF) directly on the instrument, alternated with three waits of 5 min were experimented. During syntheses, the stepwise yield of coupling reaction was automatically determined by dimethoxytrityl cations measurement. For the cleavage and deprotection step, ammonia (30%, aqueous) was used for 16 h at 60°C. After concentration *in vacuo,* molecular weight distributions of the (copolymer-ODN) conjugates were analyzed by SEC-MALLS.

RESULTS AND DISCUSSION

Strategy of Support Synthesis

P[MAMVE] and P[MAE] are alternating copolymers of maleic anhydride, with methyl vinyl ether

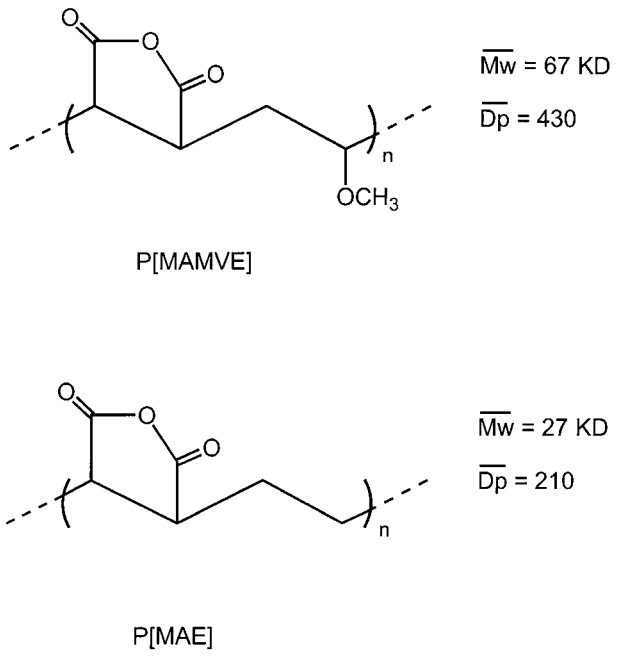


Figure 2 Chemical structure of P[MAMVE] and P[MAE].

and ethylene, respectively (Fig. 2). Their number average molecular weights ($\overline{Mn}$) of 67,000 and 27,000 correspond to ~ 430 and 210 anhydride functions per polymer chain. Anhydride moieties can easily react with both hydroxyl or amine groups to provide either ester or amide bonds. Our idea was to use the difference in stability under alkaline conditions between amide bonds and ester bonds to perform a selective cleavage of the polymer-ODN conjugate from the support. Therefore, copolymers were grafted onto silica support (CPG or glass bead) *via* alkali-labile ester bonds, and the DNA primers **I** were linked to the polymer *via* amide bonds (Fig. 3). Hence, these new supports could allow initiation of DNA chemical synthesis from primer **I.** After synthesis, a basic treatment of the support could eventually take place selectively to cleave the ester moieties and release the copolymer-ODN conjugate in solution.

5′-Dimethoxytrityl-2′-thymidine 3′-(6-aminohexyl phosphate) **I** was synthesized in two steps from thymidine. The dimethoxytrityl group at the 5′ position was removed to start DNA synthesis.

CPG and glass bead solid supports were functionalized according to the method described in the Experimental section. The first step consisted of silanization of the silica surface with an epoxy silane, with subsequent ring opening of the epoxy by HEG to yield hydroxylated particles. Quantitative titration of hydroxyl groups on the surface was achieved by their tritylation and acid-mediated detritylation with subsequent optical density measurement of the trityl cation concentration (see Experimental section). The amounts of hydroxyl groups were 16.2 μmol g^{-1} for CPG and 0.138 μmol g^{-1} for glass beads. The low specific surface area of glass beads did not allow sufficient functionalization to conduct the coupling reaction of the polymer, thus only the CPG was kept for further experiments. The HEG derivatization reaction was quantitative as the amount of epoxy groups introduced by silanization was measured to be 14.6 μmol g^{-1} (by HCl-mediated ring opening of the oxirannes and titration of the resulting hydroxyl groups by the tritylation-detritylation method). This control allowed us to confirm that there was no residual epoxide on surface after the HEG derivatization reaction.

The coupling reaction of monomer **I** onto maleic anhydride copolymers was conducted at various concentrations of **I** in anhydrous DMSO, in the presence of DMAP [0.5 Eq monomer (**I**)] (final volume: 500 μL). After reaction times of 1, 4, 24, and 48 h, 50 μL aliquots were pipetted off, diluted in 450 μL of water, and filtered before analysis by capillary zone electrophoresis. Two peaks were detected by that method, one corresponding to the reaction product and the other one to that of monomer **I.** The ratio of the areas of the peak corresponding to the conjugate to the sum of the two peaks allowed for the calculation of the coupling yield of monomer **I,** considering a potential error of ±10%. Related to the coupling yield Y is the percent of reacted anhydride group F, which can be calculated according to Equation (1)

$$F = Y \cdot E \qquad \text{(a)}$$

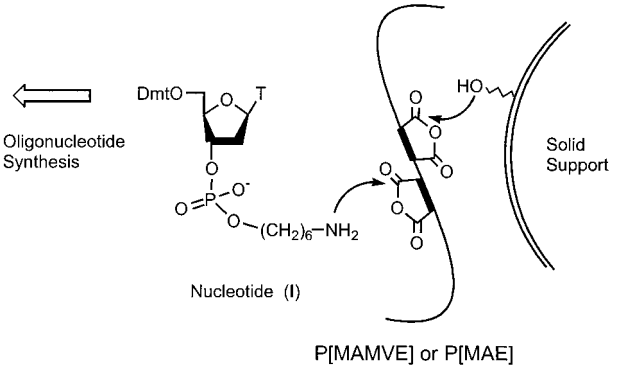


Figure 3 Principle of ODN synthesis from modified support.

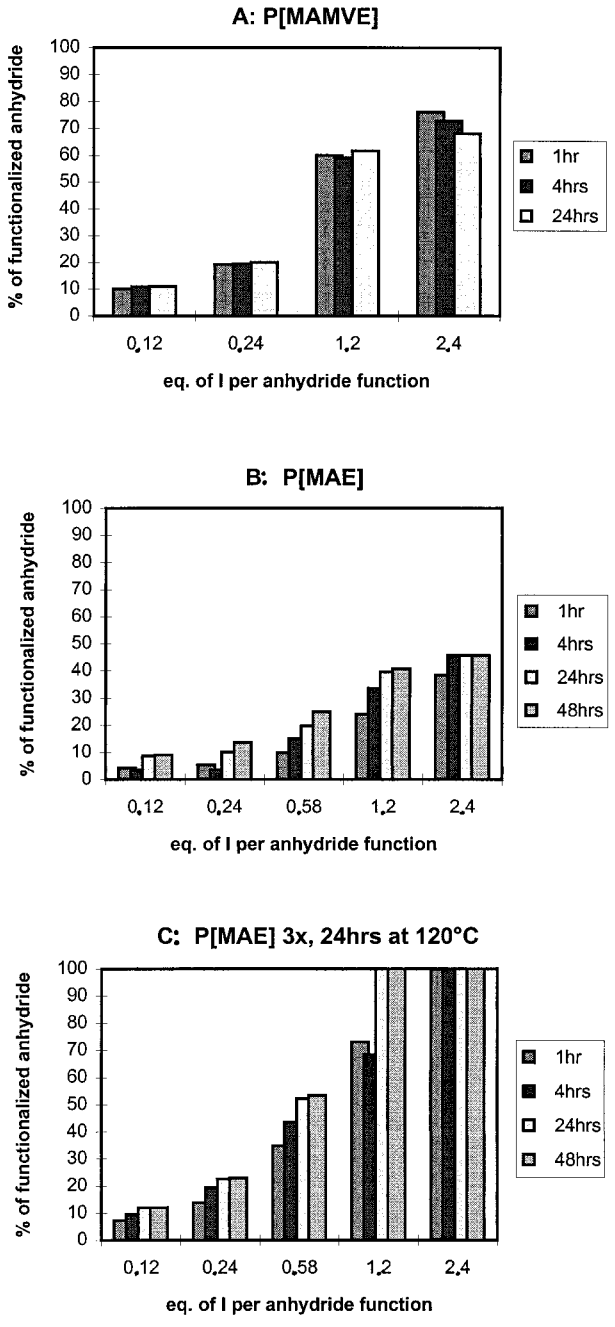


Figure 4 Percentage of polymer anhydride moieties functionalized with biomonomer **I** (*F*) *versus* number of equivalent of monomer **I** in reaction (*E*) Capillary Electrophoresis (CE) analyses.

where *E* is the initial number of equivalent of **I** per anhydride function. Graphs A, B, and C in Figure 4 shows the variation of *F versus E*. The immobilization of monomer **I** onto the P[MAMVE] was maximum after 1 h of reaction (graph A), and the percentage of functionalized anhydride moieties increased with an increase of the initial concentration in monomer **I,** to level off at 80%. Even a large excess of **I** did not allow us to reach 100% functionalization, probably because of steric hindrance brought by immobilized **I.** A more plausible explanation for the lack of completeness of the functionalization reaction was issued from the thermogravimetric analysis (TGA) of P[MAMVE]. TGA measures the loss of material with heat, and the results showed that not only did the polymer contain 0.8% free water, but also that 14% of anhydride groups were hydrolyzed under the form of a diacid. These last data prove that 100% functionalization of polymer by **I** cannot be achieved. Based on these results, conditions with 0.24 Eq of nucleotide corresponding to ~ 85 monomers per P[MAMVE] chain were selected as more favorable to preserve enough anhydrides for grafting the polymer onto the solid surface. One hour coupling time proved sufficient. With the P[MAE] copolymer, the kinetics of grafting of monomer **I** was slower than in the case of P[MAMVE], in particular at low initial concentrations of **I** where 48 h were needed for maximum functionalization. The rate of modification of the anhydride moieties leveled off at 45%. This can be regarded as a good result, because the TGA experiment showed that 48% of anhydride groups were hydrolyzed to the diacid. When P[MAE] was dried at 120°C for 24 h, before reaction with **I,** 100% of the anhydride groups on the polymer were functionalized as shown on graph C and TGA analysis run on the polymer indicated this time that anhydride groups were regenerated. Hence, to conduct grafting onto the silica solid phase, conditions with 0.24 Eq of monomer **I** and a coupling time of 4 h were selected. Polymers were kept in dried atmosphere for further experiments.

Coupling of monomer **I** to the polymer and grafting of the resulting conjugate onto the particles took place in one single step. Various conditions have been tested in particular with P[MAMVE] and coupling yields were assessed by dimethoxytrityl cation quantification (see the Experimental section). Functionalization results are reported in Table I. For experiment 1, the copolymer and monomer **I** were incubated 1 h before adding the CPG, which was allowed to react overnight. Experiments 2 and 3 were designed to assess the effect of the order of addition of the reactants: the copolymer was incubated 1 h with the CPG before adding **I.** As for experiment 1, the reaction was left overnight. For the fourth exper-

Table I Various Coupling and Grafting Reaction Experiments

Polymer	P[MAMVE]					P[MAE]
Coupling reaction experiments	1	2	3	4	5	6
Dimethoxytritye cations measurement: μmol g^{-1}	2.33	2.28	2.19	2.64	0.56	2.17

Reagents for coupling reaction with P[MAMVE]—polymer: 20 nmol; CPG: 90 mg; DMAP: 1 μmol; *N*-methyl-imidazole: 2 μmol; nucleotide: 2 μmol. Reagents for coupling reaction with P[MAE]—polymer: 60 nmol; CPG: 90 mg; DMAP: 3 μmol; nucleotide: 6 μmol. Experimental conditions—1: P[MAMVE] + nucleotide + DMAP ⇒ 1 h stirring + CPG ⇒ 16 h stirring; 2: P[MAMVE] + CPG + *N*-methyl-imidazole ⇒ 1 h stirring + nucleotide ⇒ 16 h stirring; 3: P[MAMVE] + CPG + DMAP ⇒ 1 h stirring + nucleotide ⇒ 16 h stirring; 4: P[MAMVE] + CPG + DMAP + nucleotide ⇒ 16 h stirring; 5: CPG + DMAP + nucleotide ⇒ 16 h stirring; 6: P[MAE] + nucleotide + DMAP ⇒ 4 h stirring + CPG ⇒ 16 h stirring.

iment, all reagents were mixed at the same time. Run 5 was used as a control (i.e., without any copolymer in solution). As seen in Table I, the range of functionalization was narrow (2.19–2.64 μmol g^{-1}), proving that the order of addition of the reactants had no drastic effect on the course of the reaction. Control 5 gave a low signal, indicating that a possible side reaction due to adsorption of the monomer might occur onto the solid surface. From our results, procedure 1 was preferred to conduct ODN synthesis, because the amino function of monomer **I** was allowed to react for 1 h to link polymer chains before adding CPG for grafting. With this strategy, it was postulated that the undesirable adsorption of nucleotide **I** onto the solid surface was limited.

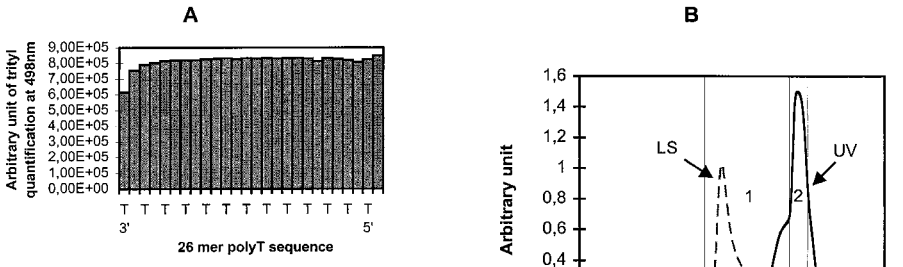
P[MAE] grafting onto the CPG surface was achieved as described in procedure 6. The course of covalent coupling of (**I**) to polymer chain was increased to 4 h, as previous studies demonstrated the slower kinetics of this reaction with P[MAE]. Reagents were also concentrated three times in the solution. Acidic treatment of the functionalized CPG led to approximately the same amount of Dimethoxytrityl Cations (DMT^+) than experiments with P[MAMVE], then corresponding to a similar amount of conjugate grafted onto the surface.

Functionalized CPGs have been analyzed in DRIFT spectroscopy. Useful spectral information could only be obtained in the IR region between 4000 and 1400 cm^{-1} because of the intense silica oxide vibrations below 1400 cm^{-1}. —CH_3 area ($\bar{\nu}$ = 3100–3000 cm^{-1}) and —CH stretching bands between 2900 and 3000 cm^{-1} were observed. More characteristic data were the ester band (1784 cm^{-1}), the carboxylic acid area ($\bar{\nu}$ = 1740–1700 cm^{-1}), and the amide band (1610 cm^{-1}), confirming the presence of the polymer on surface functionalized *via* ester and amide functions.

Oligonucleotide Synthesis

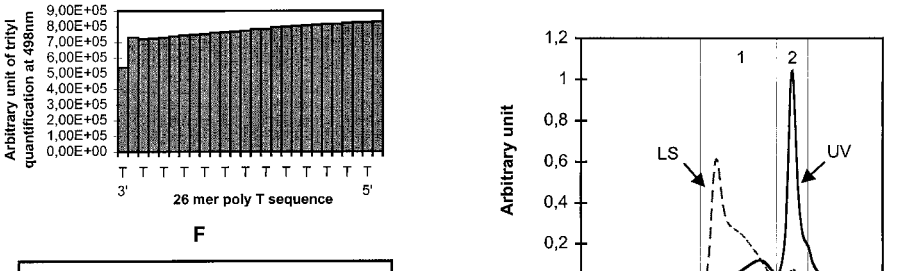
Both P[MAE] and P[MAMVE] functionalized CPGs have been tested on a expedite Millipore instrument. 26-mer polythymidine oligonucleotides were synthesized with a 0.2 μM phosphoramidite cycle program. A preliminary capping step of the support was conducted according to the following: three manual runs of 15 s each of capping cycle directly on the instrument, alternated with three waits of 5 min. This optimized capping step was used to block eventual residual hydroxyl groups on the CPG surface under the form of an inactive acetoxy group. Detritylation is a step that takes place after addition of each new base on the growing ODN. Measuring the amount of trityl cation released is a means of assessing the efficiency of the coupling step that has just occurred. On a DNA synthesizer, trityl quantification is automatic and, although units are arbitrary, these data can be compared with one another. During a standard ODN synthesis, the detritylation signal regularly decreased as the addition of a new nucleotide on the growing ODN occurred at reaction yields ranging from 97 to 99%. In our case, as shown in Figure 5(A,D) for P[MAMVE] and P[MAE], respectively, the detritylation signal increases and that occurs wherever the polymer is used.

At the end of syntheses, cleavage from P[MAMVE] and P[MAE] functionalized CPGs allowed DNA quantification by optical density measurement at 260 nm. The results showed that >100% of DNA material was recovered. One explanation for the excess product was that the capping step was not efficient; thus, hydroxyl groups were still available to start DNA elongation. Using an alkyl phosphoramidite in anhydrous acetonitrile as a capping agent lead to the same excess of products. Then, two DNA syntheses were performed: one



C

Light Scattering Calculation of $\overline{Mn}$	
$\overline{Mn}$ (UV peak 1)	82000 g.mol^{-1}
$\overline{Mn}$ (UV peak 2)	14700 g.mol^{-1}



F

Light Scattering Calculation of $\overline{Mn}$	
$\overline{Mn}$ (UV peak 1)	165000 g.mol^{-1}
$\overline{Mn}$ (UV peak 2)	14500 g.mol^{-1}

Figure 5 Characterization of (polymer-polyT 26-mer) crude syntheses. A, B, C = with P[MAMVE]; D, E, F = with P[MAE]; A, D = trityl quantification during synthesis; B, E = SEC-MALLS chromatograms; LS = light scattering signal at 90°; UV = 260 nm; C, F = number average molecular weight ($\overline{Mn}$).

with P[MAMVE] functionalized CPG, missing monomer **I** and the other with a CPG missing both monomer **I** and P[MAMVE]. In both cases, the detritylation signal increased as in Figure 5(A,D), and DNA material was recovered from the support. The main product of this crude proved to be a 25-mer polyT 3′-phosphate by Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) mass spectrometry analysis. Molecular weight distributions of the released conjugates were evaluated by a MALLS technique, coupled with a SEC system. The refractive index increment for mass calculation for DNA was 0.2 ml g^{-1} (ref. 7). Figure 5(B,E) is representative of the SEC analyses of the crudes, released from the supports after ammonia treatment. In both cases, two peaks were observed with UV detection at 260 nm; the first to elute was a broad peak ($\overline{Mn}$ 82,000 g mol^{-1} for P[MAMVE] CPG and 165,000 g mol^{-1} for P[MAE] CPG), which was assumed to correspond to the copolymer-ODN conjugate. The second peak ($\overline{Mn}$ 14,700 g mol^{-1} for P[MAMVE] CPG and 14,500 g mol^{-1} for P[MAE] CPG) could reflect the presence of either abortive conjugates or parasite ODN chains. Both peaks displayed absolutely identical UV spectra from 200 to 400 nm, identifying the nucleic acid population. The copolymer alone was never detected.

Experimental $\overline{Mn}$ of the conjugates was lower than the theoretical ones. For instance, the measured $\overline{Mn}$ for P[MAMVE]-ODN was 82,000 g

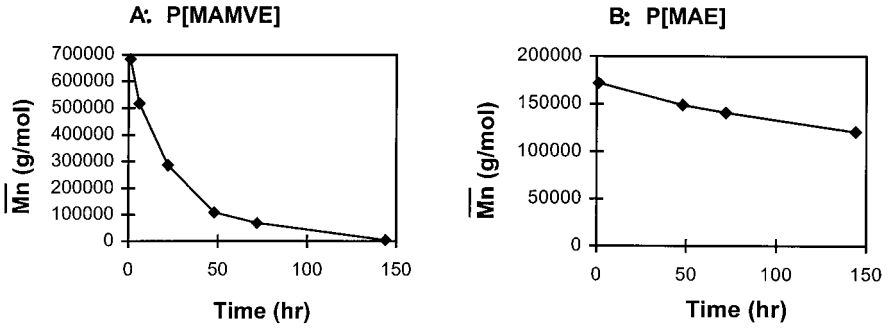


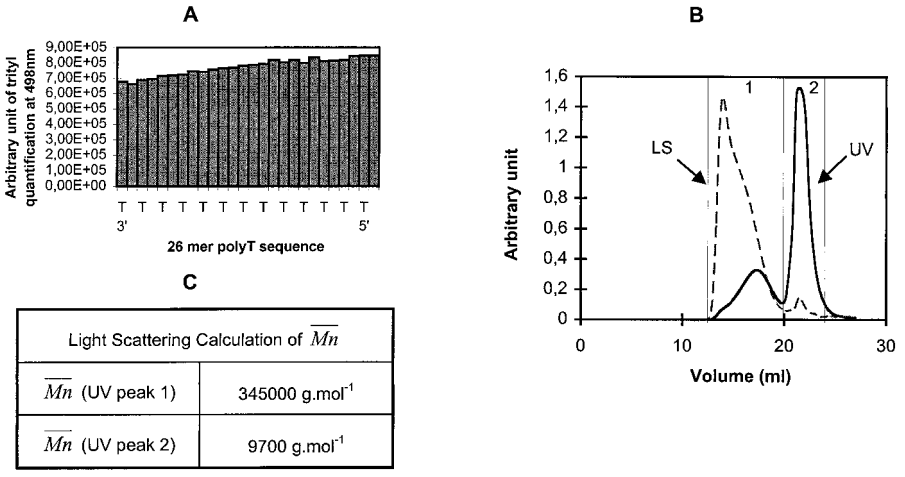
Figure 6 Number average molecular weight ($\overline{Mn}$) variation of (A) P[MAMVE] or (B) P[MAE] *versus* time of treatment in 30% NH_4OH/H_2O at 60°C.

mol^{-1} instead of 734,000 g mol^{-1} for the expected theoretical $\overline{Mn}$ considering a polyT 26-mer synthesis (calculated with a molecular weight of ODN = 7,847 g mol^{-1}, an average $\overline{Mn}$ of P[MAMVE] = 67,000 g mol^{-1}, at an overall coupling yield for DNA synthesis optimized at 100% and an estimated number of 85 ODNs per polymer chain). Three phenomena may explain such a discrepancy: (1) poor release of high molecular weight conjugates with ammonia treatment due to diffusion limitations; (2) lack of stability of the amide bonds that link the ODNs onto the polymer; and (3) degradation of the polymer chain during ammonia treatment.[16] Adding ethanol to the cleavage mixture, changing treatment time or using methylamine[17] instead of ammonium hydroxide did not improve the amount of conjugate released nor allow recovery of the compounds with the expected molecular mass. If the amide bound was not stable in the ammonia mixture, an increase in the time of exposure of the CPG from 16 to 72 h would decrease the amount and mass of the recovered polymer-ODN conjugate. SEC-MALLS analyses proved that it was not the case.

The stabilities of P[MAMVE] and P[MAE] were investigated in the standard conditions of basic treatment of synthetic oligonucleotides (30% NH_4OH/H_2O at 60°C); at various periods of time, aliquots were analyzed by SEC-MALLS. Number average molecular masses ($\overline{Mn}$) were deduced using a refractive index increment value of 0.179 ml g^{-1} for P[MAMVE] and 0.177 ml g^{-1} for P[MAE] in aqueous buffer.[18] The variation of $\overline{Mn}$ measured by SEC-MALLS *vs.* time is reported in Figure 6, showing that at t_0, in the case of P[MAMVE] [Fig. 6(A)], $\overline{Mn}$ was abnormally high. Indeed, it reflects the formation of aggregates that has already been reported.[8] Anyway, $\overline{Mn}$ continued to decrease, with time down to 4,450 g mol^{-1}, which was far below the reported value for P[MAMVE] (67,000 g mol^{-1}). This experiment confirmed the degradation of P[MAMVE] under ammonia treatment *via* a mechanism that is currently investigated.[18]

In the case of P[MAE], the same ammonia treatment leads to a different behavior [Fig. 6(B)]. A $\overline{Mn}$ decrease was indeed slighter, resulting more from a chain desaggregation process than degradation. Hence, this study confirmed the stability of P[MAE] under ammonium hydroxide treatment and corroborated SEC-MALLS $\overline{Mn}$ analysis of conjugates. Furthermore, the P[MAE]-ODN conjugate obtained after cleavage had a molecular mass closer to the expect theoretical value. The observed $\overline{Mn}$ was 165,000 g mol^{-1} instead of 435,000 g mol^{-1} for the theoretical value, assuming a P[MAE]-polyT 26-mer calculated with an average $\overline{Mn}$ of polymer = 27,000 g mol^{-1}, an ODN synthesis yield of 100%, and an estimated number of 52 ODNs per chain. Based on these results, we can regard P[MAE] as a more appropriate candidate than P[MAMVE] for the development of our alternative strategy.

Concerning the narrow peak 2 observed on UV profiles of chromatograms 5B and 5E, the average $\overline{Mn}$ obtained by light scattering calculation was in a range of 14,000–15,000 g mol^{-1} for both syntheses. These $\overline{Mn}$ data were widely influenced by peak 1 compound that was not efficiently separated by SEC. Nevertheless, low values seemed to indicate that this population might correspond to free ODN. As described earlier, nonspecific adsorption of phosphoramidite nucleosides was observed onto the glass surface, leading to excess recovered DNA material. To try to reduce this nonspecific adsorption, a new P[MAE] CPG was synthesized with P[MAE] loaded with 104 equivalents of mono-



C

Light Scattering Calculation of $\overline{Mn}$	
$\overline{Mn}$ (UV peak 1)	345000 g.mol^{-1}
$\overline{Mn}$ (UV peak 2)	9700 g.mol^{-1}

Figure 7 SEC analysis of ODN synthesis from P[MAE] CPG doubly functionalized by **I** on the polymer chain (104 **I** per chain). (A) Trityl quantification during synthesis. (B) SEC-MALLS chromatogram. LS = Light scattering signal at 90°; UV = 260 nm. (C) Number average molecular weight ($\overline{Mn}$) data.

mer **I** per polymer chain. The grafting reaction onto solid support was conducted for 48 h, leading to a final CPG that gave a trityl response of 2.6 μmol g^{-1}. A 26-mer polyT was synthesized from this support. Trityl data in Figure 7A, still increased at each successive phosphoramidite cycle, indicating that adsorption was not yet avoided. However, after 16 h of treatment with concentrated ammonium hydroxide at 60°C, the oligonucleotidic crude material was analyzed by SEC-MALLS, and the corresponding chromatogram depicted in Figure 7(B) revealed a more important conjugate to free ODN ratio. This encouraging result let us think that the way to reduce side adsorption onto silica support during oligonucleotide synthesis is to optimize the amount of biomonomer **I** covalently grafted onto the solid support through the polymer at the interface. Further experiments are in progress to confirm this statement.

CONCLUSIONS

In this article, a new route to obtain polymer-ODN conjugates was described *via* a direct synthesis of oligonucleotides onto the surface of a CPG modified by a reactive polymer. The CPG was covalently coated with an alternating copolymer (P[MAMVE]) or (P[MAE]) functionalized with aminothymidine moieties, at which site DNA elongation was initiated. 26-mer polyT ODNs were synthesized, and oligonucleotidic material was cleaved from support and deprotected through classical ammonium hydroxide treatment. SEC-MALLS analyses revealed abnormally low molecular mass for the synthetic (P[MAMVE]-polyT 26-mer) conjugate that was assigned to polymer degradation process during the cleavage using high pH conditions. On the contrary, with P[MAE], the observed molecular weight was in better agreement with the expected value. In both cases, a parasite ODN side synthesis was initiated from the support that could result from uncontrolled adsorption of the thymidine phosphoramidite synthon during ODN synthesis *via* a mechanism that is currently under investigation. Neither various capping reactions tested on CPG nor modifying cleavage conditions suppressed this side reaction. Promising results have been obtained by a twofold increase of biomonomer **I** loading on P[MAE]. This suggests that a way of reducing adsorption onto the silica surface during ODN synthesis is to limit access to this surface by increasing the amount of monomer **I** covalently tethered *via* the polymer. At this stage of our investigations, we can seriously envision synthesized conjugates with heterogeneous nucleic acid sequences with the aim of evaluating their performances in an ELOSA diagnostic test.

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