Oligonucleotide Synthesis onto Poly(*N*-acryloylmorpholine-*co*-*N*-acryloxysuccinimide): Assessment of the Resulting Conjugates in a DNA Sandwich Hybridization Test

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ABSTRACT: A water-soluble statistical poly(*N*-acryloylmorpholine-*co*-*N*-acryloxysuccinimide) [poly(NAM/NAS)] copolymer was studied for polymer–oligonucleotide (ODN) conjugate elaboration and for further use in diagnostic applications. Three different copolymers were first prepared by free-radical solution polymerization with different *N*-acryloylmorpholine (NAM) and *N*-acryloxysuccinimide (NAS) molar ratios (80/20, 70/30, and 60/40). Their number-average molecular weights ranged from 98,000 to 120,000 g/mol, as determined by aqueous size exclusion chromatography with an online light-scattering detector. Then, polymer–ODN conjugates were obtained via a strategy consisting of the direct synthesis of ODNs onto polymer chains previously grafted onto a controlled pore glass support. Before the grafting of the polymer onto the solid support, a preliminary step was performed to bind a nucleotide starter along the polymer chain (via the reactive NAS units) to initiate automated DNA synthesis. To multiply the number of ODNs growing from starters, a branched phosphoramidite synthon [bearing two *O*-dimethoxytrityl groups] was introduced at the first step of ODN elongation as a short sequence of four branched synthons alternated with three thymidine residues. Conjugates were assessed in a DNA sandwich hybridization test developed for hepatitis B virus detection. Sensitivity limits were evaluated and compared to those obtained with an other polymer, poly(maleic anhydride-*alt*-methyl vinyl ether) [poly(MA/MVE)]. A sensitivity limit of 2.6×10^7 DNA copies/mL was reached with the poly(MA/MVE)–ODN conjugate at the capture phase and with the poly(NAM/NAS)–branched ODN conjugate at the detection phase of the test. © 2004 Wiley Periodicals, Inc. J Appl Polym Sci 92: 3784–3795, 2004

Key words: DNA; solid phase synthesis; conjugate; diagnostic assay; *N*-acryloylmorpholine; hybridization

INTRODUCTION

Nucleic acids have become key molecular targets for the detection of infectious or genetic diseases. The classical *in vitro* DNA diagnostic test uses oligonucleotides (ODNs) as probes both for the extraction of the target from a biological fluid (capture step) and for its quantification through a colorimetric, or fluorescent, signal (detection step). This sandwich technique often includes amplification steps used to reach a good sensitivity level. The enzymatic amplification of the target, for instance, with the polymerase chain reaction (PCR), efficiently improves DNA detection limits. However, this very sensitive method often lacks reproducibility and requires stringent conditions to avoid contamination.[1]

As another approach, the use of branched DNA (bDNA) has also been described with the aim of the direct detection of DNA[2–4] without enzymatic amplification. This strategy affords an accurate and quantitative signal,[5,6] and intensive investigations on the reduction of nonspecific hybridization have efficiently improved the sensitivity. The last generation of a bDNA-based signal amplification assay (bDNA3.0) reaches a sensitivity limit around 10^4 DNA copies/mL. However, this kind of test requires important optimizations and is time-consuming (~24 h to completion).[7]

An alternative approach has been developed for the direct analysis of nucleic acids, with polymer–ODN conjugates used to amplify the signal in both the cap-

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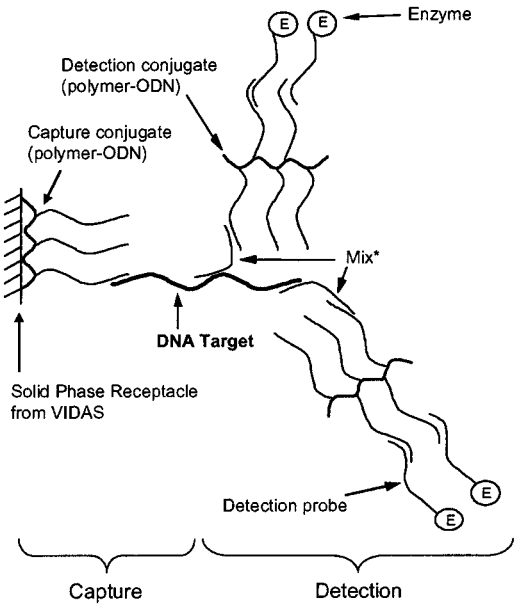

Figure 1 Principle of the ELOSA test developed for DNA detection with the polymer conjugates.

ture and detection steps of the test (Fig. 1). For instance, conjugates obtained with poly(maleic anhydride-*alt*-methyl vinyl ether) [poly(MA/MVE)] improved sensitivity when used at the capture phase.[8] In fact, such conjugates increased the amount of probe available to capture the DNA target. Also, conjugates based on poly(*N*-vinyl pyrrolidone-*co*-*N*-acryloxysuccinimide) [poly(NVP/NAS)] enhanced sensitivity when used at the detection phase.[9] Indeed, they multiplied the number of detection probes, inducing a higher colorimetric or fluorescent signal. In both cases, the conjugates were prepared via the covalent coupling of amino-bearing ODNs (independently presynthesized) onto a reactive polymer, which often resulted in aggregate formation [compact random coil; number-average molecular weight (M_n) $\approx$ 2,000,000 g/mol].[10]

Recently, a new strategy was developed for obtaining polymer–ODN conjugates. It consists of a direct synthesis of ODNs from the polymer, initiated from nucleotide starters previously bound to reactive sites along the polymer chain.[11] This strategy was first applied to maleic anhydride (MA) copolymers. Conjugates bearing numerous ODNs per chain were obtained, exhibiting a totally different structure than the conjugates classically prepared via covalent coupling; that is, the chains were not aggregated (expanded random coil; M_n = 345,000 g/mol).[10] Nevertheless, the use of such nonaggregated conjugates {from poly(maleic anhydride-*alt*-ethylene) [poly(MA/E)]} did not significantly improve the results of the diagnostic tests (in terms of the sensitivity limit).

To investigate whether other conjugates obtained via a similar straightforward synthesis strategy could offer better performances in diagnostic tests, we envisioned two main modifications: (1) a change in the nature of the polymer used as a support and (2) a multiplication of the number of ODNs bound to each reactive site along the polymer chain with a phosphoramidite branched synthon (**B**).

1. With regard to the first point, we used poly(*N*-acryloylmorpholine-*co*-*N*-acryloxysuccinimide)[12] [poly(NAM/NAS)], which differs in two main aspects from the previously used copolymers:
   - One aspect concerns the reactive site, which is an activated ester instead of an anhydride unit [in the case of poly(AM/MVE)]. Its intrinsic reactivity toward an amino-bearing counterpart should also lead to very high coupling yields, although the reaction should not be as fast. In addition, in the case of the MA copolymer, each individual coupling reaction liberates one carboxylate charge, resulting in a conjugate bearing numerous charges along the chain. On the contrary, in the case of an *N*-acryloxysuccinimide (NAS)-based copolymer, the coupling reaction will not produce any charge, and the corresponding conjugate will bear only a few charges arising from the hydrolysis of residual NAS units.
   - The other aspect concerns the nature of the comonomer. For poly(MA/MVE) and poly(MA/E), the comonomer (methyl vinyl ether or ethylene) is slightly or not hydrophilic, whereas for poly(NAM/NAS), the comonomer *N*-acryloylmorpholine (NAM) is very hydrophilic. In fact, NAM is a bisubstituted acrylamide derivative that presents several interesting features,[12] in particular, an ability to produce high-molecular-weight polymers that are soluble in water and in polar or low-polar solvents.[13] We anticipated that such differences between the comonomers would influence the synthesis of the polymer–ODN conjugates, their structure and conformation, and hence, their performances in enzyme-linked oligosorbent assay (ELOSA) diagnostic tests.
2. With regard to the second modification, that is, the use of a phosphoramidite **B**, the strategy was identical to that developed for bDNA synthesis,[4] except that, in our case, the branched ODNs were bound to a polymer chain (Fig. 2).

In fact, **B** was introduced onto the polymer–nucleotide starter **1** conjugate at the first step of ODN elongation, followed by a short sequence that alternated **B** and the thymidine synthon (**T**). At each incorporation, this **B** created a kind of fork, multiplying

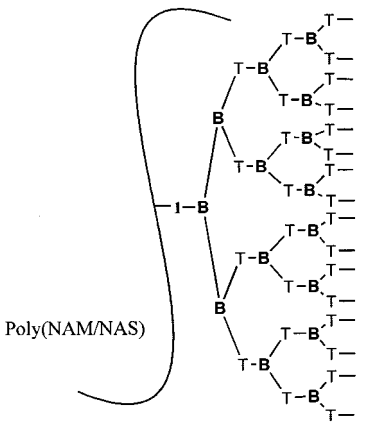

Figure 2 Branched sequence *1BBTBTBT*.

(by 16 in theory) the numbers of ODNs that subsequently grew from the polymer. This strategy led to the production of high-molecular-weight conjugates bearing numerous ODNs.

In this article, we describe as a first part the direct synthesis strategy onto the poly(NAM/NAS) copolymer. Several copolymers were used with differing NAM/NAS molar ratios (80/20, 70/30, and 60/40). The resulting polymer–ODN conjugates were compared with poly(MA/E)–ODN conjugates. Then, they were evaluated in a hepatitis B virus (HBV) DNA sandwich hybridization assay, performed on a bioMérieux's VIDAS immunoanalysis instrument (in either the capture or detection phase). Second, the synthesis of **B** and the resulting poly(NAM/NAS)–branched ODN conjugates are reported, together with their assessment in a similar HBV diagnostic test. Finally, the sensitivity results obtained from the different strategies are discussed.

EXPERIMENTAL

Reagents and analyses

The controlled pore glass (CPG; mean pore diameter = 2000 Å, particle size = 40–85 μm, surface area = 9.2 m^2/g) was ordered from Fluka (Buchs, Switzerland). Anhydrous dimethyl sulfoxide (DMSO; 99.9+%) and dimethylformamide (DMF; 99.8%) were purchased from Aldrich (Saint Quentin Fallavier, France). Dimethoxytrityl chloride (95%, Aldrich), 2-cyanoethyl *N,N'*-diisopropylchlorophosphoramidite (Aldrich), hexaethylene glycol (97%, Aldrich), diisopropylethylamine (99%, Aldrich), DMAP (99%, Aldrich), triethylamine (99.5%, Aldrich), *p*-toluenesulfonic acid monohydrate (98%, Aldrich), glycidyloxypropyltrimethoxysilane (97%, Fluka), and fluorescamine (Acros, Noisy-Le-Grand, France) were used as received. ODN1 was ordered from Eurogentec (Seraing, Belgium).

Nucleotide starter **1** was synthesized as previously described.[10]

NAM (99%, Polysciences, Inc., Warrington, PA) was purified by distillation under reduced pressure. NAS (98%, Acros) was purified by silica gel chromatography (the eluent was ethyl acetate/methylene chloride at 6/4 v/v). Azobisisobutyronitrile (AIBN; 98%, Merck, Fontenay-Sous-Bois, France), used as the polymerization initiator, was purified by recrystallization in ethanol. Dioxane (99.5%, SDS, Peypin, France), used as a polymerization solvent, was purified by distillation over $LiAlH_4$.

Poly(MA/MVE) was obtained from Polysciences. Its M_n (67,000 g/mol) was determined by size exclusion chromatography (SEC) in DMSO at 80°C (Shodex mixed column, 25 cm) with poly(ethylene oxide) (POE) standards for the calibration.[14]

The molecular weights and molecular weight distributions of the poly(NAM/NAS) copolymers were determined with a light-scattering apparatus [three-angles laser-light-scattering (TALLS) photometer from Wyatt Technologies, Santa Barbara, CA] associated with a differential refractometer (DRI Waters 410, Saint Quentin Yvelines, France) as an online double detection for SEC analysis. Analyzes were performed by the injection of 100 μL of polymer solution (conc. = 5×10^{-3} g/mL) in an injection loop connected to a Waters 510 pump and Waters UltraHydrogel 2000 and 500 columns. The eluent was a borate buffer (0.05 mol/L, pH 9.3) at a flow rate of 0.5 mL/min. The specific refractive index increment of poly(NAM/NAS) was determined with the same eluent and with a Brice Phoenix differential refractometer equipped with a filtered white light source at 530 nm (Phoenix, AZ). A value of 0.162 mL/g was obtained.

The purity of the polymer–ODN conjugates were determined by SEC with a Waters UltraHydrogel 500-Å column, a Kontron HPLC 422 pump, a Kontron HPLC autosampler 465, and a Kontron diode array detector 440. The eluent was a phosphate buffer (0.1*M*, pH 6.8) at a flow rate of 0.5 mL/min. Ultraviolet (UV) detection was achieved at 260 nm.

Fluorescence measurements were run on a PerkinElmer LS 50 (Wellesley, MA). High-resolution mass spectrometry (HRMS) analyses were performed on a ZAB 2-SEQ instrument (VG Analytical, Manchester, UK). ^{1}H-NMR and ^{31}P-NMR spectra were recorded on an Bruker Avance 200 MHz spectrometer (Wissembourg, France. Capillary zone electrophoresis was carried out on an Applied Biosystems instrument equipped with a silica capillary (72 cm $\times$ 50 μm, Applera, Courtaboeuf, France). The analysis was run in a sodium carbonate buffer (20 m*M*) with UV detection at 260 nm. ODNs were synthesized on an Applied Biosystems DNA synthesizer (model 394) by β-cyanoethyl phosphoramidite chemistry.

TABLE I
Initial NAM and NAS Concentrations Used for Radical-Initiated Copolymerization in Dioxane Solution at 60°C

Sample	[NAM] (mol/L)	[NAS] (mol/L)
Poly(NAM/NAS)$_{(80/20)}$	0.810	0.200
Poly(NAM/NAS)$_{(70/30)}$	0.703	0.300
Poly(NAM/NAS)$_{(60/40)}$	0.590	0.400

[AIBN] = 0.005 mol/L.

Synthesis of the poly(NAM/NAS) copolymers

Polymerization experiments were performed in a three-necked bottomed flask equipped with a condenser, a magnetic stirrer, and a nitrogen inlet. The reaction vessel was loaded with dioxane (49 mL) and the comonomer mixture (Table I) and was purged with nitrogen for 1 h at 20°C. Then, temperature was raised to 60°C with a thermostated oil bath. Finally, the initiator (preliminary dissolved in 1 mL of dioxane) was added to the reaction mixture, and the copolymerization was carried out under a nitrogen atmosphere for 2 h. Then, the polymerization mixture was poured into a large volume of diethyl ether (1000 mL) to precipitate the copolymer and to remove any residual monomer.

Synthesis of poly(MA/MVE)–ODN conjugate [1]

Conjugate [1] was obtained according to the protocol described in ref. 11. Dry ODN1 (15 nmol; bearing an amino arm at its 5′ extremity) was dissolved in 7 μL of 0.1*M* sodium borate/0.5*M* sodium chloride buffer (pH 9.3). Poly(MA/MVE) (0.07 mg) dissolved in 153 μL of DMSO was added, and the reaction was carried out for 3 h at 37°C. The solvents were then removed *in vacuo*, and the conjugate was resuspended in 200 μL of water. Conjugate [1] was used as described for ELOSA assays.

Synthesis and purification of poly(NAM/NAS)–ODN conjugates [2], [3], [4], and [5]

Derivatization of the CPG beads

Hydroxylated CPG was obtained by the protocol described by Maskos and Southern.[15] First, CPG was functionalized by silanization with 3-glycidyloxypropyltrimethoxysilane. In the second step, the epoxide residue reacted with hexaethylene glycol. The amount of available hydroxyl functions on the surface was estimated by the dimethoxytritylation titration method around 17–20 μmol/g.[11] The hydroxyl-derivatized support was stored under a dried atmosphere at room temperature without any further precautions.

Coupling of the nucleotide starter **1** onto poly(NAM/NAS)$_{(80/20)}$

The polymer (70.4 mg; corresponding to 95.8 μmol of NAS units) was dissolved in 1 mL of DMF. In parallel, **1** (10 mg, or 13.8 μmol) was dissolved in 1 mL of the same solvent. Then, the polymer solution (30 μL; 2.9 μmol of NAS units) was mixed with 348 μL of DMF, 0.1 mg of DMAP (0.84 μmol), and 122 μL (1.68 μmol) of **1** to get a total volume of 500 μL. The mixture was stirred for 6 days at room temperature. We determined the coupling yield by fluorescence titration following a specific reaction between the amine group of residual **1** and fluorescamine. For instance, 50 μL of the reaction mixture was titrated by 150 μL of a fluorescamine solution in DMF (0.3 mg/mL). After 5 h of storage under darkness, the fluorescence intensity was measured at 470 nm (excitation wavelength = 393 nm). The fluorescence intensity was related to the concentration of residual **1**, with a calibration curve established under the same conditions. No fluorescence was detected in a control sample without starter. The coupling yield was 87%.

Coupling of **1** onto poly(NAM/NAS)$_{(70/30)}$

The copolymer (47.8 mg; 95.8 μmol of NAS functions) was dissolved in 1 mL of DMF. The coupling of **1** on poly(NAM/NAS)$_{(70/30)}$ was then achieved, as described previously. The coupling yield was 85%.

Coupling of **1** onto poly(NAM/NAS)$_{(60/40)}$

The copolymer (36.6 mg; 95.8 μmol of NAS functions) was dissolved in 1 mL of DMF. The coupling of **1** on poly(NAM/NAS)$_{(60/40)}$ was then achieved, as described previously. The coupling yield was 84%.

Grafting of the poly(NAM/NAS)–1 conjugate onto hydroxylated CPG beads

Hydroxylated CPG (100 mg) was added to each of the previous three coupling solutions, and mixtures were stirred at room temperature for 6 additional days. Fully derivatized CPG was then efficiently washed with DMF and acetone before it was dried *in vacuo*. The efficiency of the poly(NAM/NAS)–**1** conjugate grafting onto the CPG beads was estimated by the quantification of dimethoxytrityl cation (Dmt^+) groups that were released from CPG under acidic treatment.[11] For instance, 5 mg of CPG was poured into 1 mL of 3% trichloroacetic acid (TCA) in CH_2Cl_2. After 5 min, the mixture was diluted with 4 mL of 0.1*M* *p*-toluenesulfonic acid monohydrate in acetonitrile, and the optical density was measured at 498 nm (ε_{Dmt+} = 70 mL μmol^{-1} cm^{-1}). The Dmt^+ concentration corresponded to the amount of nucleotide **1**

(bound to the polymer) at the CPG surface, and therefore, the measure could be correlated to the conjugate loading on the support. By this method, loadings of poly(NAM/NAS)$_{(80/20)}$–nucleotide **1**, poly(NAM/NAS)$_{(70/30)}$–nucleotide **1**, and poly(NAM/NAS)$_{(60/40)}$–nucleotide **1** were determined to be 3.1, 3.9, and 4.5 μmol of nucleotide **1**/g of CPG, respectively.

Synthesis of conjugates [2], [3], [4], and [5]

Conjugates [2], [3], [4], and [5] were synthesized from the fully derivatized CPG with the following general protocol:

Each of the different poly(NAM/NAS)–**1**-derivatized CPGs (30 mg) was used to develop ODN synthesis with a standard 1-μmol DNA cycle. Before ODN elongation, acetic anhydride capping of the CPG surface with the two capping solutions [acetic anhydride/pyridine/tetrahydrofuran (THF) and *N*-methylimidazole/THF; Applied Biosystems] was performed directly in the synthesizer with four runs of 10 s, alternated with three pauses of 5 min. ODN2 or ODN3 sequences were performed, with an average coupling of 98% per cycle. After synthesis, poly(NAM/NAS)–ODN conjugates were recovered in 4 mL of concentrated ammonia solution (30% w/w) for 16 h at 60°C. After drying *in vacuo*, hydrolysis products were suspended in 500 μL of water, and the conjugates were purified by filtration with Centricon 100 device (Millipore Corp. (Billerica, MA); membrane cutoff = 100,000 g/mol). Twelve successive concentration and dilution cycles led to the separation of conjugate from undesirable products of lower molecular weight. After purification, conjugate purity was calculated from an SEC chromatogram by comparison of the conjugate and impurity peak areas. The purity of conjugates [2], [3], [4], and [5] were estimated as 98, 95, 89, and 90%, respectively.

TABLE II
Description of the Various ODN Sequences

Reference	Sequence
ODN1	5′ TCAATCTCGGGAATCTCAATGTTAG 3′
ODN2	5′ TCAATCTCGGGAATCTCAATGTTAGTTTT 3′
ODN3	5′ AACGCTACTACTATTAGTAGTTTT 3′
ODN4	5′ AACGCTACTACTATTAGTAG 3′

Synthesis and purification of poly(NAM/NAS)–ODN branched conjugate [6]

Synthesis of 1,4-*O*-bis(4,4′-dimethoxytrityl)-1,2,4-butanetriol (**A**)

1,2,4-Butanetriol (0.68 mL, or 7.6 mmol) and DMAP (134 mg, or 1.1 mmol) were coevaporated twice with 10 mL of anhydrous pyridine and were then dissolved in 40 mL of the same solvent. One equivalent (7.6 mmol) of *N,N*-diisopropylethylamine and 2.3 equiv (17.3 mmol) of dimethoxytrityl chloride were successively added to the solution and stirred overnight at room temperature under nitrogen atmosphere. After concentration *in vacuo*, the mixture was dissolved in 200 mL of CH_2Cl_2. The organic phase was successively washed with 100 mL of saturated $NaHCO_3$ aqueous solution, washed twice with 100 mL of water, dried over $MgSO_4$, and evaporated to dryness *in vacuo*. The residue was purified by column chromatography (dichloromethane/cyclohexane at 50/50 v/v; 0.5% triethylamine (TEA)) to produce **A** with a 52% yield:

HRMS Calcd for $C_{46}H_{46}O_7$ $[M+Li]^+$: 717.3446 g/mol. Found: 717.3404 g/mol.

^{1}H-NMR (δ, ppm, $CDCl_3$): 1.76 (q, 2H, CHOH—*CH*$_2$—CH$_2$), 3–3.3 (m, 4H, O—*CH*$_2$—CHOH—CH$_2$—*CH*$_2$—O), 3.77 and 3.79 (2s, 2 × 3H, O—CH$_3$), 4 (m, 1H, *CH*OH), 6.7–7.5 (m, 26H, aromatic).

Synthesis of 1,4-*O*-bis(4,4′-dimethoxytrityl)-2-*O*-(2-cyanoethyl-*N,N*′-diisopropyl aminophosphinyl)-1,2,4-butanetriol (**B**)

A (0.5 mg, or 0.7 mmol) and DMAP (12 mg, or 0.098 mmol) were coevaporated twice with 3 mL of dried pyridine and twice with 3 mL of dried THF. The mixture was then dissolved in 5 mL of dried THF, and 244 μL (1.4 mmol) of *N,N*-diisopropylethylamine was added to the medium. 2-Cyanoethyl *N,N*′-diisopropylchlorophosphoramidite (178 μL, or 0.8 mmol) was added to the stirred solution (within 10 min) under a nitrogen atmosphere, and the reaction was carried out for 4 h at room temperature. CH_2Cl_2 (50 mL) was added, and the organic phase was washed once with 40 mL of saturated $NaHCO_3$ aqueous solution and twice with 40 mL of water and was then dried over $MgSO_4$. The organic phase was then evaporated to dryness *in vacuo*. The product **B** was purified by silica gel chromatography (ethyl acetate/*n*-hexane = 15/85 v/v; 0.5% TEA). A pure fraction of **B** was concentrated and then resuspended in 20 mL of benzene, filtrated, and lyophilized. Pure **B** was recovered at an 88% yield.

HRMS Calcd for $C_{55}H_{63}O_8N_2P$ $[M+Li]^+$: 917.4497 g/mol. Found: 917.4482 g/mol.

^{31}P-NMR (δ, ppm, CD_3CN): 143.78 and 143.85 (isomers).

Poly(NAM/NAS)$_{(70/30)}$–ODN4 branched conjugate [6] synthesis

The previously described poly(NAM/NAS)$_{(70/30)}$–nucleotide starter **1** conjugate-derivatized CPG (20 mg; 3.9 μmol of **1**/g of CPG) was used for branched conjugate synthesis. First, the sequence 3′ **1BB**T**B**T**B**T 5′ was synthesized with a 0.1*M* solution of **B** in dry

TABLE III
Characterization of Poly(NAM/NAS) Copolymers

Sample	Conversion (%)[a] NAM	Conversion (%)[a] NAS	Copolymer Composition: NAM/NAS[b]	*Mn* (g/mol)[c]	PDI[c]
$Poly(NAM/NAS)_{(80/20)}$	94.6	98.3	79.6/20.4	98200	1.8
$Poly(NAM/NAS)_{(70/30)}$	89.0	91.7	69.5/30.5	112300	1.8
$Poly(NAM/NAS)_{(60/40)}$	82.5	81.7	59.8/40.2	121200	2.0

[a] Determined by ^{1}H-NMR.[18]
[b] Obtained from the initial monomer concentrations (Table I) and the individual monomer conversions.
[c] SEC measurement with TALLS detection.

acetonitrile. The standard 1 μM phosphoramidite DNA cycle program was used with an additional wait of 15 s at the coupling step. The coupling yield per cycle was measured after a manual collection of TCA/CH_2Cl_2 solutions and Dmt^+ quantification through dilution in a 0.1*M* toluene sulfonic acid/acetonitrile solution. The average coupling yield of **B** was 70%. ODN4 (HBV detection sequence, Table II) was then synthesized from the branch extremities with a global phosphoramidite coupling yield of 98%. The cleavage of the conjugates from the support and the deprotection of nucleic base amino groups were performed in 1*M* NaOH for 48 h. Branched conjugate solutions were then desalted through a controlled pore size membrane (Centricon 3, membrane cutoff = 3000 g/mol) and were further purified by five successive filtrations on a Centricon 100 membrane (cutoff = 100,000 g/mol). Branched conjugates were then purified up to 100% by SEC as follows. The sample was injected on an 500-Å UltraHydrogel column, and the fraction corresponding to the pure conjugate was collected. After several injections, conjugate fractions were then desalted and concentrated by filtration on a Centricon 100 membrane to keep [6] in a final volume of 500 μL of water.

Estimation of ODN number per polymer chain

The number of ODNs grown from the polymer chain was approximated for the conjugates with the following equations.

For conjugates [2], [3], [4], and [5], the oligonucleotide number bound to the chain (N) was

$$N = N_{NAS} \times E \times C \times T^4 \quad (1)$$

where N_{NAS} is the average *N*-acryloxysuccinimide number per polymer chain, as calculated with M_n [see Table III; N_{NAS} = 133 for $poly(NAM/NAS)_{(80/20)}$, 225 for $poly(NAM/NAS)_{(70/30)}$, and 318 for $poly(NAM/NAS)_{(60/40)}$]; E is the equivalent number of starter **1** per NAS unit (E = 0.58); C is the coupling yield of **1** to the polymer; and T is the average coupling yield of **T** during the 3′ TTTT 5′ automated synthesis (T = 0.98). C was the value obtained by the titration of residual **1** in solution after 48 h of the coupling reaction, as described in the Experimental part. The possible additional binding of **1** onto the polymer chain during the 6 more days of incubation necessary for polymer grafting onto CPG was considered negligible.

For this equation, N was estimated as 100 for [2] and [4], 60 for [3], and 140 for [5] (Table IV).

However, we used the coupling yield of phosphoramidite synthons during ODN synthesis to calculate a full size (complete sequence) ODN number bound to the polymer with

$$N_{min} = N \times S^n \quad (2)$$

where N_{min} is the minimum number of full-size ODNs bound to the polymer, S is the average coupling yield

TABLE IV
Principal Characteristics of the Conjugates

Conjugate	Polymer nature	ODN	Conjugate purity (%)	ODN per polymer[a]
[1]	Poly(MA/MVE)	ODN1	88	13
[2]	$Poly(NAM/NAS)_{(70/30)}$	ODN2	98	60–100
[3]	$Poly(NAM/NAS)_{(80/20)}$	ODN3	95	40–60
[4]	$Poly(NAM/NAS)_{(70/30)}$	ODN3	89	65–100
[5]	$Poly(NAM/NAS)_{(60/40)}$	ODN3	90	95–140
[6][b]	$Poly(NAM/NAS)_{(70/30)}$	ODN4	100	265–395

[a] Estimated number of ODN per polymer chain as calculated with M_n of Table III.
[b] Branched conjugate.

of phosphoramidite synthons during ODN synthesis ($S = 0.98$), and n is number of bases per ODN.

For this equation, N_{min} was estimated as 60 for [2], 40 for [3], 65 for [4], and 95 for [5].

For branched conjugate [6]

$$N = N_{NAS} \times E \times C \times (2 \times B)^4 \times T^3 \quad (3)$$

where B is the average coupling yield of **B** during the 3′ T*BB*T*B*T*B*T 5′ synthesis ($B = 0.7$) and T is the average coupling yield of **T** during the 3′ T*BB*T*BT*B*T* 5′ synthesis ($T = 0.98$).

For the branched conjugate [6], N was estimated at around 395 [$N_{NAS} \times 0.58 \times 0.85 \times (2 \times 0.7)^4 \times 0.94$]. N_{min} was calculated with eq. (2).

For conjugate [1] [obtained by coupling the 5′ amino linker ODN1 to poly(MA/MVE)], the coupling yield was estimated as 88% from the SEC chromatogram, which corresponded to 13 ODNs bound to the chain.

Detection of HBV DNA by the ELOSA test

This test was performed on a bioMérieux's VIDAS immunoanalysis instrument. The target used was a PCR product of HBV DNA (double-stranded 2339 bp) cloned in pBR 322 and purified by agarose gel electrophoresis, as previously described by Erout et al.[9] The test was based on a sandwich hybridization system[16,17] with an additional step with 17 specific probes complementary to highly conserved regions of the HBV DNA sequence.[10]

At the capture phase, conjugate [1] or [2] was coated (1 h at 37°C) on the inside of a disposable conical polystyrene–polybutadiene solid-phase receptacle (SPR) by passive adsorption.[10] The conjugate coating concentration (corresponding to the concentration of ODNs bound to the polymer chain) was 150 n*M* in phosphate buffered saline (PBS). After this coating step, hybridization with the target was performed at 37°C for 45 min in a poly(ethylene glycol) (PEG) buffer. Then, a solution of 17 ODNs (mix) complementary to the HBV sequence was added in PEG buffer.[10] These 17 ODN bore at their 5′ extremity an identical non-HBV sequence of 20 nucleotides (complementary to ODN4) that could specifically bind to the detection conjugates at the following detection step (Fig. 1). Conjugates [3]–[6] were diluted to 15 n*M* in PEG buffer and used at this step as previously described.[10] At last, an ODN probe bearing an enzyme (alkaline phosphatase) at its 3′ extremity was introduced for specific hybridization with the detection conjugate. The enzyme substrate (4-methyl-umbelliferyl phosphate) led to a fluorescent product whose signal was expressed in relative fluorescence unit (RFU) by the VIDAS detector.

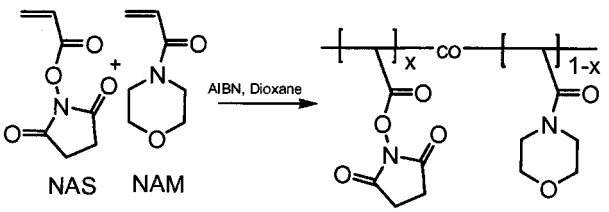

Figure 3 Synthesis of the poly(NAM/NAS) copolymer.

RESULTS AND DISCUSSION

Synthesis of the poly(NAM/NAS) copolymers

Several studies were recently reported on the polymerization of the NAM/NAS comonomer pair. First, an optimization of their free-radical solution copolymerization was carried out by variation of different parameters, including the monomer concentration, the initiator (AIBN)/monomer ratio, and the nature of the solvent.[13] Dioxane appeared to be the best solvent for the synthesis of high-molecular-weight polymer chains (up to 100,000 g/mol), with a total monomer concentration of 1 mol/L and an initiator/monomer molar ratio of 0.5% (Fig. 3).[13]

A kinetic study of the free-radical solution copolymerization was also performed with different NAM/NAS molar ratios.[18] For low NAS feed compositions (20 and 30%), NAS copolymerized slightly faster than NAM. For the particular case of the 60/40 NAM/NAS molar ratio, both monomers polymerized at the same rate, which gave rise to copolymers of constant composition during the whole polymerization time. In fact, the determined reactivity ratios[18] ($r_{NAS} = 0.63$ and $r_{NAM} = 0.75$) indicated that whatever the comonomer feed ratio was, the variation of the copolymer composition versus conversion was very limited, especially below 90% conversion. This comonomer pair copolymerized statistically, and consequently, the obtained copolymer chains exhibited very homogeneous compositions.

The characteristics of the poly(NAM/NAS) copolymers used in this study are given in Table III. The molecular weights determined by aqueous SEC with an online light-scattering detector corresponded to absolute values.

The M_n values of the various copolymers ranged between 98,000 and 120,000 g/mol, which was the targeted molecular weight, and the polydispersity index (PDI) was close to 2, a classical value for conventional radical polymerization. Due to the hydrophilic feature of the NAM monomer, the obtained copolymers were soluble in a wide range of solvents (e.g., chloroform, dioxane, DMF, DMSO).[19] Further, because of the activated ester carried by the NAS units, the copolymers presented numerous reactive sites per chain. In addition, the reactive activated ester units were well spaced along the polymer chains, as re-

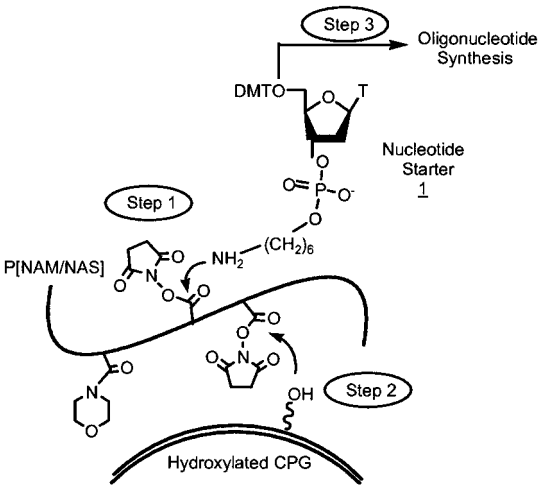

Figure 4 Principle of the polymer–ODN conjugate direct synthesis.

vealed by a ^{13}C-NMR microstructure study. This should have favored both the fixation of the nucleotide starter onto the chains and the growth of the ODNs by decreasing stearic interactions.

Conjugate synthesis strategy

Derivatization of CPG support

The strategy to produce poly(NAM/NAS)–ODN conjugates developed herein was already been described with MA copolymers [i.e., poly(MA/MVE), poly(MA/E)].[11] It consisted of the total synthesis of ODN fragments from a polymer chain previously grafted onto CPG support. The elaboration of the polymer-modified CPG was achieved in several steps from hydroxylated beads.

The first step consisted of the coupling of the nucleotide starter **1** (Fig. 4) onto the polymer chain in an organic solvent. As described in the Experimental part, 0.58 equiv of **1** per succinimide ester function (NAS unit) was introduced. We performed a kinetic study by capillary electrophoresis for poly(NAM/NAS)$_{(70/30)}$ to follow the increase in the coupling yield from the nucleotide-starter peak disappearance on the electrophoregrams (Fig. 5).

After 4 days, the coupling reaction reached 82%. After 6 days, the identification of the residual nucleotide-starter peak was difficult because of the detector sensitivity limit. This measure did not allow us to accurately access to the coupling yield value. We estimated the value as 85% by another technique, that is, a fluorescence titration consisting of a specific reaction between the residual nucleotide starter and fluorescamine, as described in the Experimental part. By this same fluorescence titration, the coupling yields of nucleotide **1** onto poly(NAM/NAS) (80/20) and (60/40) were 87 and 83%, respectively, after 6 days of the reaction, which revealed a similar reactivity of nucleotide **1** toward the activated ester of polymers made with different NAM/NAS monomer ratios. As a comparison, the nucleotide **1** coupling onto MA copolymers appeared to be more efficient because the reaction yielded 85% in 1 h with poly(MA/MVE) and 100% in 24 h with poly(MA/E)[11] with the same conditions. This result probably reflects the higher reactivity of MA compared to succinimide function toward amine compounds. Moreover, different polymer rearrangements in the DMF solvent may further explain the observed kinetics.

In the second step, hydroxylated CPG was added to the previous solutions, which were gently stirred for 6 more days (step 2, Fig. 4). After washing, the nucleotide-starter loading on CPG was determined via spectrometric quantification of the Dmt^+ released from the beads by an acidic treatment (3% TCA/CH_2Cl_2). The higher the NAS composition in the polymer was, the higher the nucleotide-starter amount (Dmt^+ titration) was per gram of CPG [3.1 μmol/g for the poly(NAM/NAS)$_{(80/20)}$, 3.9 μmol/g for the (70/30) and 4.5 μmol/g for the (60/40)]. In fact, as the amount of **1** bound to the polymer increased proportionally to the NAS ratio in the chain (0.58 equiv of **1**/NAS unit introduced in the coupling reaction) increased; the resulting nucleotide-starter loading onto CPG increased the same way. These loading values were close to those obtained for poly(MA/E) (1.5–4.25 μmol/g), according to the quantities of nucleotide coupled to the polymer.[10]

Synthesis and characterization of the poly(NAM/NAS)–ODN conjugates

In a third step, standard 1-μmol ODN synthesis columns adapted to the Applied Biosystems instrument were loaded with the derivatized CPG. A capping step with acetic anhydride was performed, as described in the Experimental part, to block residual hydroxyl groups on the CPG surface, which could initiate un-

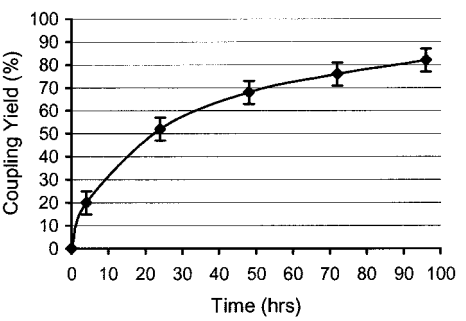

Figure 5 Electrophoresis analysis of the nucleotide starter **1** coupling reaction onto a poly(NAM/NAS)$_{(70/30)}$ capillary.

controlled ODN syntheses. Appropriate ODN sequences (ODN2 or ODN3, Table II) were synthesized with the 1-μmol phosphoramidite cycle. ODN2 included the sequence of the capture probe of the HBV ELOSA test, followed by four additional thymidines at the 3′ end to space the ODN from the polymer. In the same manner, ODN3 corresponded to the complementary sequence of the detection probe with four additional thymidines at the 3′ extremity.

The coupling yield per cycle monitored on the synthesizer averaged 98%. After the complete elongation of the ODN, the polymer–ODN conjugates were released in solution by ammonia treatment. Indeed, this basic treatment induced the selective cleavage of ester bonds tethering the polymer chain to the CPG support. After the elimination of ammonia *in vacuo*, the conjugates were purified by filtration on a controlled pore size membrane (cutoff = 100,000 g/mol) and analyzed by SEC [Fig. 6(B)].

As shown in Figure 6(A), the product eluted from 9 to 15 min (30%) corresponded to the conjugate. The

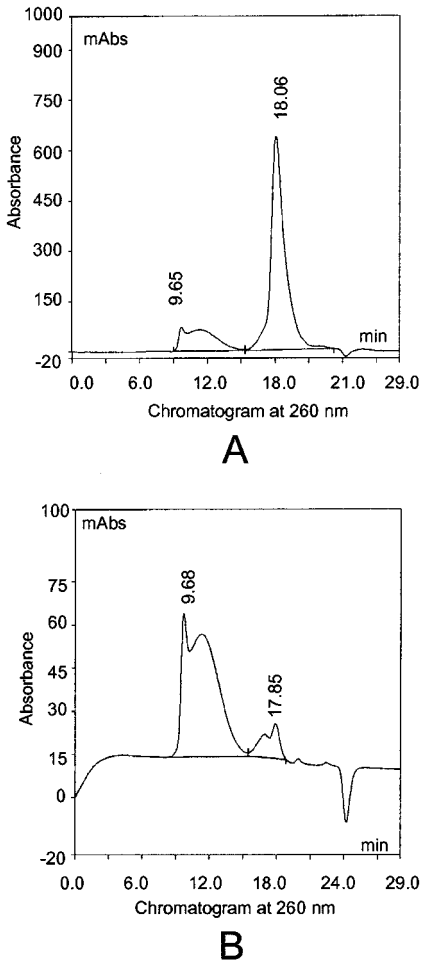

Figure 6 Chromatograms of conjugate [4] (A) before and (B) after purification.

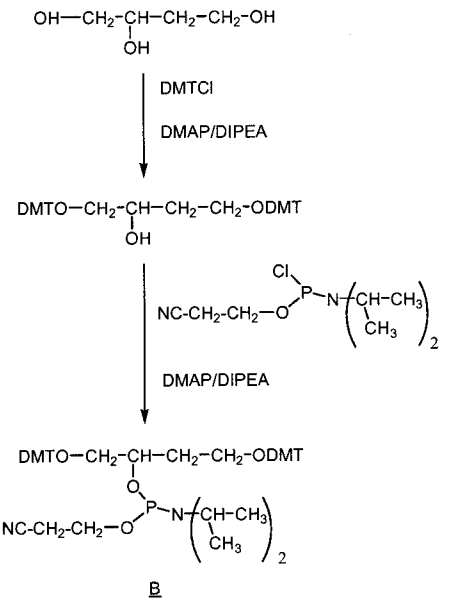

Figure 7 Synthesis of the branched phosphoramidite **B**. DIPEA = *N*,*N*-diisopropylethylamine; DMTCI = dimethoxytrityl chloride.

second product (15–22 min, 70%) was attributed to a parasite ODN population because the corresponding UV spectrum (220–400 nm) appeared to be characteristic of nucleic acid material. This second population might have resulted from different origins. Some ODNs could have been cleaved from the polymer chain during ammonia treatment. Also, uncontrolled ODN syntheses could have been initiated from the CPG because of a partial capping of residual hydroxyl groups by the acetic anhydride treatment. In response to the first hypothesis, the stability of the purified conjugate in 30% aqueous NH_4OH at 60°C was evaluated. After 48 h, around 20% of the ODNs were cleaved from the poly(NAM/NAS) chain (seen as the appearance of a second peak on the SEC chromatogram). Thus, with the assumption that the hydrolysis kinetics were the same for the pure conjugate and the conjugate bound to the CPG, probably no more than 10% of the ODNs were cleaved from the polymer after the 16-h treatment. Therefore, conjugate degradation could not have been the only cause for a parasite ODN population of such importance (i.e., more than 50% according to the peaks areas). The second hypothesis appeared equally valuable. Indeed, we had already observed a similar parasite ODN population during ODN synthesis onto poly(MA/E) copolymer.[11] Further specific investigations have lately shown that the parasite syntheses could be initiated from the uncapped hydroxyls of the CPG (unpublished results). However, after purification, the conjugate purity reached 90% [Fig. 6(B)].

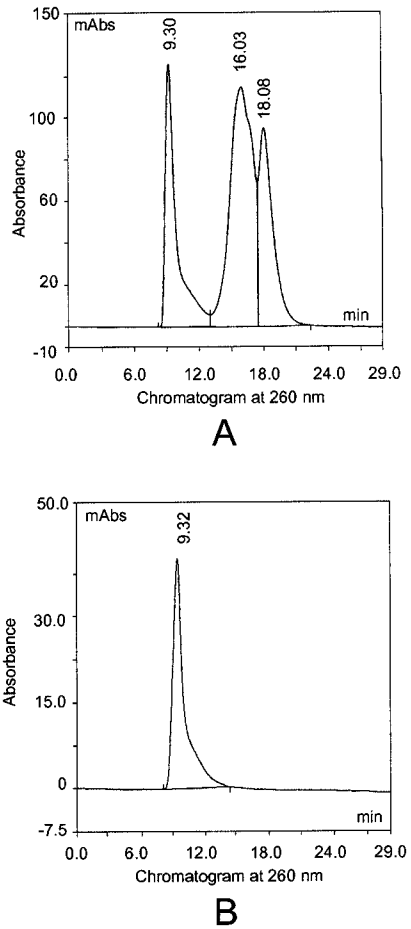

Figure 8 Chromatograms of conjugate [6] (A) before and (B) after purification.

Synthesis of the branched conjugate poly(NAM/NAS)$_{(70/30)}$–ODN4

Synthesis of the phosphoramidite **B**

The phosphoramidite **B** was obtained in two steps from 1,2,4-butanetriol (Fig. 7). First, the dimethoxytrityl protecting group was introduced on the two primary alcohol functions of 1,2,4-butanetriol. Then, a reactive phosphoramidite was substituted on the residual secondary hydroxyl, as described in the Experimental part. The global yield of the synthesis was 45.8%. The use of this **B** at the beginning of an ODN synthesis resulted in the appearance of a fork that should have doubled, at each elongation step with **B**, the number of ODNs initiated from only one nucleotide starter (Fig. 2).

A short sequence including **B**s and **T**s (3′ *BB*T*B*T*B*T 5′) was developed from the poly(NAM/NAS)$_{70/30}$-derivatized CPG with the ODN synthesizer. This sequence included four **B**s that could have theoretically multiplied by a factor of 2^4 the possible ODN number per starter molecule. A coupling yield of 70% was measured for each **B** cycle. Three **T**s were also inserted in the sequence to space the **B**s with a yield of 98%.

Then, the detection probe ODN (ODN4) was developed from the branches with a standard 1-μM DNA cycle. Phophoramidite coupling yields appeared to be identical to those obtained during linear ODN synthesis. Branched conjugates were cleaved from the support with NaOH 1N for 48 h. These drastic conditions were necessary because the classical ammonia treatment was not sufficient for the total release of branched conjugate from the support. The crude mixture was analyzed by SEC [Fig. 8(A)].

The product eluted from 8 to 13 min corresponded to the branched conjugate. The second population (from 13 to 18 min) was attributed to an ODN of high molecular weight (branched structure). The third population, eluted from 18 to 22 min, was attributed to smaller molecules such as truncated sequences because filtration on a Centricon 100 membrane (cutoff = 100,000 g/mol) selectively eliminated this latter population but not the second one. The stability of the purified conjugate in 1*N* NaOH was evaluated by SEC. After 72 h, around 6% of the ODNs were cleaved from poly(NAM/NAS). Then, only a low degradation of conjugate could have occurred after 48 h of the same treatment. Consequently, the second product could have resulted from ODN syntheses directly initiated from CPG because of a deficient capping, as previously observed for linear conjugate synthesis.

To carry out diagnostic assays, we purified the branched conjugate to 100%, as described in the Experimental part [Fig. 8(B)]. The principle characteristics of the various conjugates are reported in Table IV.

Assessment of the various conjugate performances in an ELOSA test run on the VIDAS instrument

The HBV test protocol (described in the Experimental part) used as target a double-stranded HBV DNA with 2339 base pairs and amplified by PCR. Before the assay, the DNA was denatured with 0.2*M* NaOH to separate both strands in solution. The ODN probes were selected to be specific of highly conserved re-

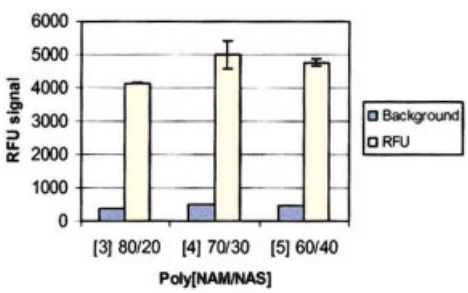

Figure 9 ELOSA signal obtained with conjugates [3], [4], and [5] at the detection step and with a constant DNA target concentration (10^{10} copies/mL). The mean values of duplicate measurements are given. The background was a whole test without a DNA target.

TABLE V
Sensitivity Limits of ELOSA Tests

Test	Capture/ detection conjugates	Blank RFU signal[a]	Sensitivity limit (signal/cutoff = 1)
1	[2]/[4]	225	4.2×10^8 DNA copies/mL
2	[2]/[6]	580	1.5×10^8 DNA copies/mL
3	[1]/[4]	113	1.8×10^8 DNA copies/mL
4	[1]/[6]	168	2.6×10^7 DNA copies/mL
5	ON[b]/[4]	30	1.2×10^9 DNA copies/mL
6	ON[b]/[6]	300	3.7×10^9 DNA copies/mL

[a] Average of four blanks (Full test − DNA target).
[b] ODN capture probe.

gions of the HBV DNA. The common sequence corresponding to ODN1 and ODN2 was used for the capture step, and the common sequence of ODN3 and ODN4 was used for the detection step (Table II). The whole sandwich assay is shown in Figure 1.

The first experiment was run with a constant DNA target concentration of 10^{10} copies/mL to compare the three different conjugates, [3], [4], and [5], at the detection phase. A poly(MA/MVE)–ODN conjugate [1] was used at the capture phase (Fig. 9) because it was described as the best candidate for amplifying the signal of HBV diagnostic tests run on the VIDAS instrument.[10]

All of the conjugates synthesized from poly(NAM/NAS) efficiently amplified the detection signal, but although each of them bore different ODN numbers along the polymer chain (Table IV), the obtained RFU signals were not significantly different. No clear influence of the conjugate structure was evidenced on the signal amplification efficiency.

Nevertheless, we preferred the conjugate giving the best signal (i.e., conjugate [4]) to run the experiment to determine the sensitivity limit of the test. A set of diluted HBV DNA targets (from 10^7 to 10^{10} copies/mL) was used for the assays. Four blanks were run first (the full test without the DNA target), and cutoff values were calculated with the following equation:[9]

$$\text{Cutoff} = \text{Average blank signal} + 3\times \text{Standard deviation} \quad (4)$$

The sensitivity limit of the assay was fixed at a signal/cutoff ratio of 1.

Six different tests were run. Tests 1 and 2 were developed with poly(NAM/NAS) conjugate [2] at the capture phase and either poly(NAM/NAS) conjugate [4] (test 1) or branched poly(NAM/NAS) conjugate [6] (test 2) at the detection phase. Tests 3 and 4 used the same two conjugates at the detection phase but used poly(MA/MVE) conjugate [1] obtained by the coupling method at the capture phase. Tests 5 and 6 were the control runs, with free ODN probe at the capture phase (Table V).

Figure 10 shows sensitivity results of the tests. In comparison with the two controls, assays with conjugates at both the capture and detection phases revealed a better sensitivity limit ($<5 \times 10^8$ DNA copies/mL; Table V). Conjugate [1] appeared to be the best candidate at the capture phase. This result was in agreement with previous studies demonstrating the great efficiency of the poly(MA/MVE) conjugate at capturing the DNA target as it formed aggregates, which favored adsorption onto the SPR.[10] However, conjugates [4] and [6], synthesized from poly(NAM/NAS), provided good results at the detection step. Furthermore, branched conjugate [6] led to a sensitivity limit three times better than conjugate [4] when associated with capture conjugate [2] and seven times better than conjugate [4] when associated with [1]. It allow a limit of 2.6×10^7 copies/mL when used with [1] without an increase in the background signal (i.e., the blank value, which corresponded to the full test without a DNA target; 168 RFU). In comparison with results previously described with this kind of assay on

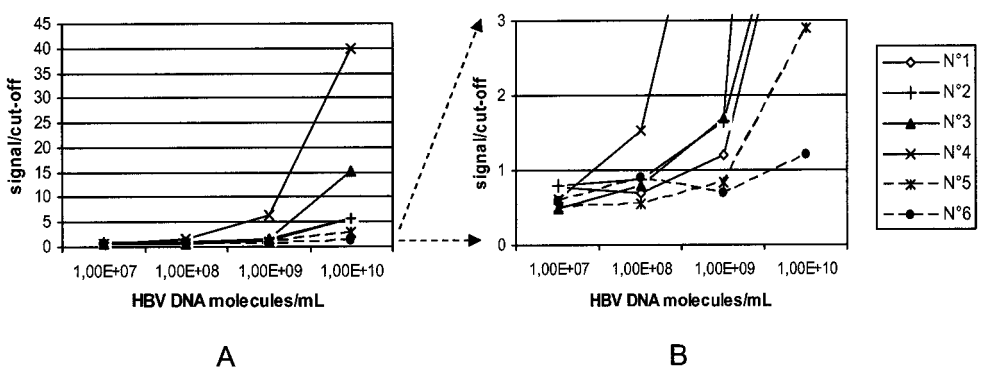

Figure 10 Detection of HBV DNA target with conjugates at both the capture and detection phases. The six assays are described in Table V. (A) Whole results and (B) enlargement of part (A) for the low-values range that was used to estimate the sensitivity of the different systems. A ratio of greater than 1 signifies a positive result.

the VIDAS instrument, the sensitivity limit reached here was a bit more efficient than the one obtained with conjugate poly(NVP/NAS)–ODN) at the detection phase (i.e., 5×10^7 copies/mL of HBV DNA).[9]

As the branched conjugate led to such amplification of the signal when used at the detection part, other branched poly(NAM/NAS) conjugates bearing the ODN capture sequence were synthesized and evaluated for capture amplification. Unfortunately, preliminary experiments run at 10^{10} copies/mL showed the background signal of the blank as important as the one of the whole test (~10,000 RFU). This result could be explained by some unspecific adsorption on the SPR of either the detection conjugate or the enzyme-labeled probe, after the coating of the branched capture conjugate on surface. Therefore, for signal amplification at the capture step, the branched structure obtained with the poly(NAM/NAS) conjugate did not appear to be as efficient as the aggregated structure obtained with the poly(MA/MVE) conjugate synthesized via the coupling method.

CONCLUSIONS

The poly(NAM/NAS) copolymer appeared to be a good candidate for the elaboration of polymer–ODN conjugates for sandwich hybridization assays. Free-radical polymerization allowed us to obtain polymers of high molecular weight ($M_n \approx 100{,}000$ g/mol) and to control NAM/NAS ratios in the chain. The activated ester functions borne by the NAS units efficiently reacted with amino counterparts, in our case the nucleotide starter **1**, with coupling yields reaching close to 90% after 6 days of reaction. Poly(NAM/NAS) was well adapted to grow ODN probes from the polymer chain, as described in this article. The resulting poly(NAM/NAS)–ODN conjugates were more stable in ammonia than those obtained from MA-based copolymers. SEC chromatograms demonstrated the higher quality of the conjugate syntheses (compared to conjugates with MA-based copolymers), even if uncontrolled parasite ODN syntheses were still initiated from the support by residual uncapped hydroxyl arms on the CPG surface. Experiments are in progress to improve this capping step. Poly(NAM/NAS)–ODN conjugates were purified up to 90% by several filtrations and dilutions on controlled pore size membranes (cutoff = 100,000 g/mol). Conjugates enhanced the signal of the HBV ELOSA test run on bioMérieux's VIDAS instrument when used at both the capture and detection steps. To increase the number of ODN probes bound to the polymer chain, a phosphoramidite **B** was introduced at the beginning of ODN synthesis as a short sequence of **B** and **T**s. The resulting poly(NAM/NAS)–branched ODN conjugates gave rise to an additional improvement in the test sensitivity limits. With a combination of poly(MA/MVE)–ODN conjugates obtained by a coupling method at the capture step and poly(NAM/NAS)–branched ODN conjugates at the detection step, the sensitivity limit reached 2.6×10^7 DNA copies/mL.

In a continuous effort to further improve the sensitivity of ELOSA-type diagnostic tests, we are currently trying to increase the capture probability of the DNA target by designing new polymer–ODN conjugate architectures. For instance, amphiphilic block copolymers based on poly(NAM/NAS) could be an advantageous candidate, with the hydrophobic block providing strong immobilization of the conjugate onto hydrophobic supports and favoring the extension of the hydrophilic part (bearing the DNA probes) into the aqueous phase, thus improving the accessibility toward DNA targets. Such block copolymers have been synthesized in our laboratory[19,20] with a recent controlled radical polymerization process, the reversible addition–fragmentation chain transfer.

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