Syntheses of Poly[*N*-(2,2 dimethoxyethyl)-*N*-methyl acrylamide] for the Immobilization of Oligonucleotides

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SYNOPSIS

Radical-initiated polymerization of *N*-(2,2 dimethoxyethyl)-*N*-methylacrylamide has been carried out either in chloroform or methanol using 2,2′-azobisisobutyronitrile as an initiator, allowing us to prepare acetal containing water-soluble polymers. A kinetic study in both solvents showed that this monomer fairly homopolymerized ($k_p \cdot k_t^{-1/2} = 1$ mol$^{-1/2}$ L$^{1/2}$ s$^{-1/2}$). Static light scattering was used to characterize the molecular weight of these polymers. In addition, the Mark–Houwink–Sakurada relationship was established based on viscosity measurements performed at 25°C in water. Recovery of the aldehyde moieties on the polymer was achieved under mild conditions using a diluted inorganic solution. The analysis of the formation of aldehyde groups was performed by ^{1}H- and ^{13}C-NMR. The covalent binding of oligodeoxyribonucleotides was carried out in water/acetonitrile mixtures with subsequent $NaBH_4$ reduction of the imine bonds so as to stabilize the polymer/oligodeoxynucleotide conjugates. © 1996 John Wiley & Sons, Inc.

INTRODUCTION

Binding oligodeoxynucleotides (ODN) to water-soluble synthetic polymers can be useful in various diagnostics applications, in order to increase the coating efficiency on a solid support[1] or for use as a detection signal amplifier.[2,3] In previous work from this laboratory, we demonstrated the suitability of maleic anhydride copolymers[2,3] and of *N*-vinylpyrrolidone/*N*-acryloxysuccinimide (NVP/NAS) copolymers[1,2,4] for the binding of nucleic acid probes bearing a primary amino group at the 5′ position. The immobilization of ODN onto these synthetic macromolecules relied on the formation of an amide bond by nucleophilic attack, by the primary amino group of the probe, on either activated *N*-hydroxysuccinimide esters in the case of NVP–NAS copolymers, or anhydride rings with maleic anhydride derivatives.

One of the main advantages of these polymers relies on the fact that they do not require any activation prior to coupling of amino-functionalized biomolecules. But hydrolysis of the activated moieties (esters or anhydride) is the major competitive reaction since the immobilization step occurs in a partially aqueous medium.

Furthermore, after completion of the coupling step and hydrolysis of the remaining groups, the resulting product is negatively charged, due to the carboxylate ions coming from the hydrolysis of either *N*-hydroxysuccinimide esters or anhydride rings. This negative net charge could be involved in nonspecific interactions in further applications. Therefore, there was a need to study the immobilization of ODN onto uncharged water-soluble polymers, bearing functional groups susceptible of forming covalent bonds with 5′ aminated DNA fragments.

Schauer et al.[5] studied the copolymerization of methacryl aldehyde with hydrophilic comonomers, but they noticed a spontaneous intramolecular cyclization of adjacent aldehyde groups directly bound to the backbone of the polymer, preventing any use later on for the coupling of biomolecules.

Zàbransky et al.[6,7] reported a thorough study on crosslinked polyacetal gels of acryl and methacrylamide derivatives. From their results, it turns out that

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- *N*-disubstituted methacrylamide acetals do not undergo any solution homopolymerization due to steric strain with the methyl group on the C—C double bond.
- products obtained by homopolymerization of *N*-disubstituted acrylamide acetals were more thermally stable than were the *N*-monosubstituted counterparts and, moreover, gave no secondary reactions with the aldehyde groups recovered after acidic hydrolysis of the acetals.

Interesting results on the covalent immobilization of enzymes onto crosslinked gels of acrylamidoacetaldehyde were obtained by Epton et al.,[8] proving that this monomer could provide material capable of forming covalent bonds with molecules of biological interest. Furthermore, acryl or methacryl amidoacetaldehyde derivatives have been used in patents[9] for the syntheses of latex particles bearing functional groups for the binding of biomolecules.

Based on these encouraging results, we decided to investigate the synthesis of noncrosslinked water-soluble polymers with *N*-(2,2-dimethoxyethyl)-*N*-methyl acrylamide and their use in the immobilization of ODNs for diagnostic applications.

EXPERIMENTAL

Materials and Methods

Chloroform from Janssen and methanol from SDS were distilled over calcium hydride before use. 2,2′-Azobisisobutyronitrile from Merck was recrystallized from ethanol from SDS and stored under nitrogen at +04°C.

Unless stated otherwise, chemicals were used as received. Infrared spectra were recorded on a Nicolet 5 PC FTIR equipment. ^{1}H-NMR spectra, 200 MHz, were recorded on a Brucker AC 200 equipment and 500 MHz spectra on a Varian spectrometer. ^{1}H-NMR and ^{13}C-NMR refer to tetramethylsilane as an internal standard. ^{13}C were recorded on the Brucker AC 200 equipment (50 MHz in ^{13}C) or the Varian spectrometer (125 MHz).

Gas chromatography analyses were performed on a Perkin-Elmer auto system, with a flame ionization detector and a Perkin-Elmer Nelson 1020 GC plus integrator.

Synthesis of *N*-(2,2 dimethoxyethyl)-*N*-methyl Acrylamide (DNMA)

The monomer synthesis was achieved according to Ref. 6: yield 82% after distillation under reduced pressure in the presence of barium carbonate and hydroquinone.

IR spectrum (cm^{-1}): 2941; 2835; 1653.

^{1}H-NMR, $CDCl_3$, 25°C (500 MHz) (ppm): 6.59 (m, 1 H, H1); 6.29 (m, 1 H, H2); 5.64 (m, 1 H, H3); 4.50 and 4.37 (t, ^{3}J4,5 (5.4 Hz), 1 H, H4, 4′); 3.48 and 3.43 (d, ^{3}J4,5 (5.4 Hz), 2 H, H5, 5′), 3.37 (s, 6 H, H6); 3.1 and 3.01 (s, 3 H, H7, 7′).

^{13}C-NMR $CDCl_3$, 25°C (125 MHz): 166.9–166.5 (C1, 1′); 128–127.7 (C2, 2′); 127–127.4 (C3, 3′); 103.3–103.1 (C4, 4′); 55–54.7 (C5, 5′); 52.2–50.4 (C6, 6′); 37.2–35.1 (C7, 7′).

Homopolymerizations

Polymerization reactions were carried out either in degassed chloroform or in methanol at 60°C; 2,2′-azobisisobutyronitrile (AIBN) was used as an initiator. After completion of the reaction, the polymers were purified by concentration under reduced pressure and then by several precipitations in ethyl ether (runs 1–9, Table I) or once with 150 mL of ether for runs 10–13. Kinetics were monitored by GC using a carbowax column (T injection = T detection = 280°C; oven temperature: 200°C; nitrogen flow: 10 mL min^{-1}) or by ^{1}H-NMR by monitoring the variation with time of the ratio of the peak area of vinyl protons at 5.64 ppm vs. the peak area of the acetal protons at 4.5 and 4.37 ppm. The latter peak area remains constant throughout the polymerization reaction and so it can be used as an internal standard to assess the consumption of the vinyl protons during the polymerization process (Fig. 1).

Polymer analyses were performed using an IR spectrometer as well as ^{1}H- and ^{13}C-NMR. Molecular weights were determined by static light-scattering experiments in DMF (SDS) with a Brookhaven instrument equipped with a 2 W laser Model 2560 (Spectra-Physics) (ionized argon) and using a sample cell dipped in a decalin bath. The specific refractive index increment (dn/dc) was obtained in the same solvent by differential refractometry (Brice-Phoenix) and, as such, $dn/dc = 0.069 \pm 0.003$ mL g^{-1} (value descended from five polymers). Light-scattering data were exploited so as to calculate the weight-average molecular weight ($\overline{M_w}$) and second virial coefficient (A_2). Viscosity studies were carried out at 25°C in water using a capillary viscosimeter (Viscosimatic VCD, Amtec).

^{13}C-NMR of a fully protected homopolymer in D_6 DMSO at 60°C (50 MHz) (ppm): 174 (C1); 103–101.7 (C4); 54.1–53 (C6); 35.7 (C7); 35.7–34.4 (C3 and C2).

Table I Solution Homopolymerizations: Conditions and Results

Run Code	Polymer	Solvent	DNMA (mol L^{-1})	Overall Conversion (%)	Yield (%)
1	P101	$CHCl_3$	0.158	66	33
2	P102	$CHCl_3$	0.226	80	66
3	P103	$CHCl_3$	0.404	86	72
4	P104	MeOH	0.226		40
5	P105	MeOH	0.415	93	63
6	P106	MeOH	0.552		65
7	P107	MeOH	0.64		76
8	P108	MeOH	0.581	100	85
9	P109	MeOH	0.92		
10	P110	MeOH	0.391	100	26
11	P111	MeOH	0.583	100	40
12	P112	MeOH	0.792	100	41
13	P113	MeOH	1.156	100	70

Polymer Deprotection

To recover the aldehyde groups on the polymer, deprotection was performed under various acidic conditions, in the presence of water and of an organic solvent, either at room temperature or under reflux. The yield of deprotection was obtained by calculating the area ratio of the ^{1}H-NMR aldehyde peak at 9.4 ppm vs. the acetal peak centered at 4.5 ppm. ^{13}C-NMR was used to check the disappearance of the acetal carbon at 101.7–103 ppm and the appearance of the aldehyde carbon at 197.8 ppm.

^{13}C-NMR and DEPT135 of a fully deprotected homopolymer, D_6 DMSO, 60°C (50 MHz): 197.8 (C4); 174 (C1); 57.5 (C6); 37.8–33.7 (C2, C3, C7).

Oligonucleotide (ODN) Coupling

Oligodeoxynucleotides were synthesized at the Laboratoire des Sondes Froides, bioMérieux, with a Model 394 Applied Biosystem synthesizer, using cyanoethyl phosphoramidite chemistry according to the manufacturer's protocols. Unless stated other-

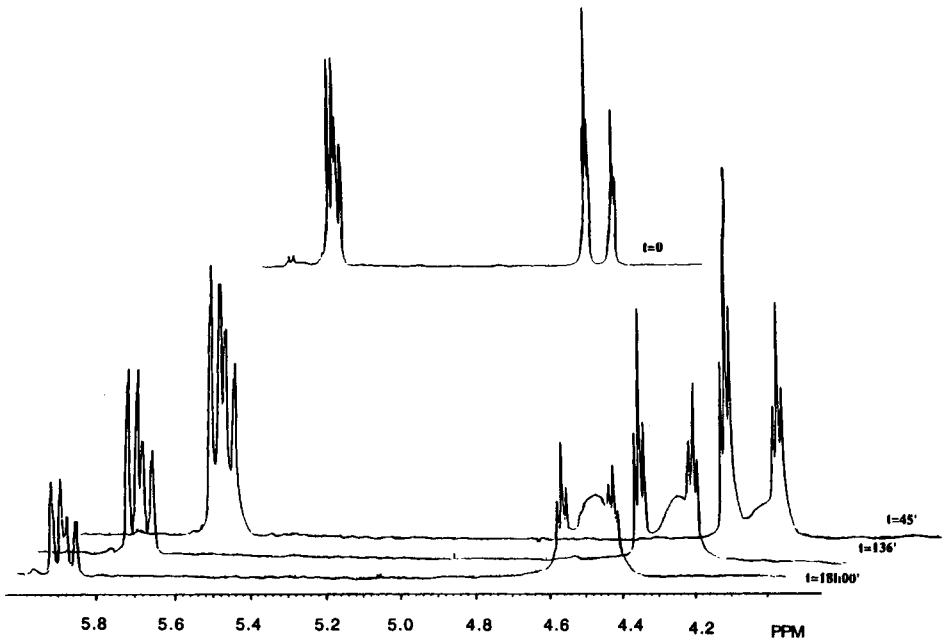

Figure 1 Evolution of ^{1}H-NMR spectrum of reaction mixture vs. time for homopolymerization of DNMA (run 3).

wise, every oligonucleotide probe bears an Applied Biosystem Aminolink II spacer arm (aminohexamethylene spacer); the following sequences were used (given from positions 5′ to 3′) and used without further purification:

Sequence A: GAT GAG CTA TAT GAG AAC GGT A

Sequence B: AAC GCT ACT ACT ATT AGT AG

Sequence C: 13 mer poly(dT)

Sequence D: 20 mer poly(dT)

Sequence E: 32 mer poly(dT).

Coupling reactions with deprotected polymers were performed at 37°C for various periods of time in a water/acetonitrile mixture containing diisopropylethylamine (DIEA) as a catalyst. When necessary, the reduction of the Schiff base was achieved by adding into the reaction mixture 16 μL of a 8 mg mL^{-1} aqueous solution of sodium borohydride or sodium cyanoborohydride.

Crude products were purified by high-performance liquid chromatography (HPLC) (Kontron) using a Waters Ultra-Hydrogel 500 column and a 0.1*M* phosphate buffer, pH 6.8. Detection was achieved with a UV detector by measuring the optical density at 260 nm, corresponding to the ODN adsorption. The ratio of the peak area, corresponding to the bound oligonucleotides, vs. the peak area of the free, uncoupled oligonucleotide gave the coupling yield referring to the initial amount of ODN. The polymer has no adsorption at the considered wavelength and concentration.

RESULTS AND DISCUSSION

Monomer Synthesis (DNMA)

The synthesis of the monomer was straightforward and gave high yields of the desired product after distillation. ^{1}H-NMR shows that the product is a 60/40 mixture of conformers A and A′ (Fig. 2) since the peaks for the acetal proton, *N*-methyl protons, and *N*-methylene protons are split. With a methyl group being less bulky than is a dimethoxyethyl group, A is probably the major conformer, where the carbon–carbon double bond is *E* (*s-trans*), referring to the C—N bond, to the dimethoxyethyl group. The ^{13}C spectrum displays split peaks for all carbon atoms, but the acetal, *N*-methyl and *N*-methylene carbons are the most affected due to the proximity of the C—N bond. These carbons correspond to the positions where we had noticed the most important ^{1}H signal splitting.

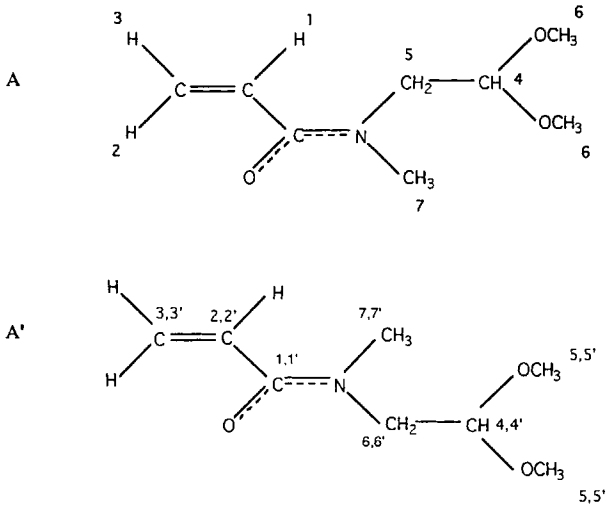

Figure 2 DNMA monomer: conformers A and B. Conformer A with ^{1}H-NMR numbering. Conformer B with ^{13}C-NMR numbering.

Homopolymerizations and Polymer Characterizations

Radical-initiated homopolymerizations were carried out in solution either in chloroform or in methanol. Polymerization conditions and results are summarized in Table I.

Polymerization Conditions and Kinetics

The effect of the monomer concentration on the yield of recovered dry polymer after precipitation is particularly marked for polymerizations that take place in chloroform. Nevertheless, whatever the solvent, on increasing the monomer concentration, the yield in recovered material increases far more than the conversion (which represents the monomer consumption) does. It proves that for the low initial monomer concentration the precipitation conditions are not efficient enough to recover all polymer chains formed during the reaction (the low $\overline{M_w}$ being left in solution). When the average polymerization degree of the chain increases, the precipitation in diethyl ether is more efficient and the yield increases.

^{1}H-NMR was used to monitor the kinetics of homopolymerizations run in chloroform (Fig. 1). In Figure 3, the overall monomer conversion vs. time for runs 1 and 2 is plotted; one can see that the larger the monomer concentration, the higher the conversion. It is worth noting that, although the initial polymerization rate is fairly high, neither of

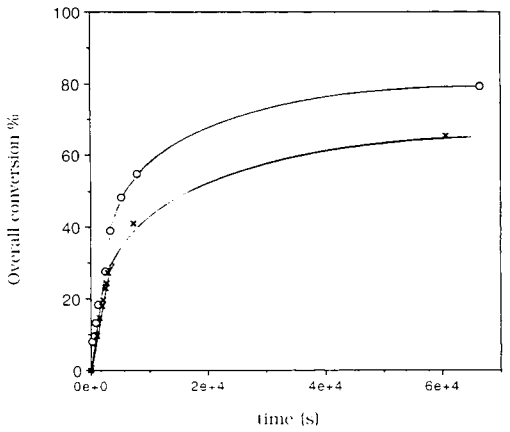

Figure 3 Homopolymerization kinetics of DNMA, runs (O) 1 and (x) 2 in chloroform (Table I) from ^{1}H-NMR data.

these two experiments reaches 100% conversion. The ratio of the propagation rate constant vs. the squared root of the termination rate constant, $k_p \cdot k_t^{-1/2}$, can be obtained by plotting Ln ([Mo]/[M]) vs. $1 - \exp(-k_d \cdot t^{1/2})$ (see Fig. 4) and the values are of 1 and 1.07 $mol^{-1/2}\ L^{1/2}\ s^{-1/2}$ for runs 1 and 2, respectively, considering a k_d value of AIBN in DMF at 60°C of $0.85 \cdot 10^{-5}\ s^{-1}$.[10] These values, around 1, are independent from monomer concentrations and denote the good polymerizability of the monomer.

NMR was not appropriate to monitor the kinetics of polymerizations in methanol due to solvent peak interferences; thus gas chromatography (GC) was used, with DMSO as an internal standard, and for run 8, the ratio $k_p \cdot k_t^{-1/2}$ was 1.035 $mol^{-1/2}\ L^{1/2}\ s^{-1/2}$. Monomer conversions were found higher in methanol than in chloroform. In runs where DMSO was introduced to serve as an internal standard, it interfered with the precipitation step of the polymer, reducing the yields in recovered material.

Polymer Molecular Weight Analysis

The molecular weight determination was achieved by static light scattering of polymer solutions in DMF using the value of *dn/dc* as reported in the Experimental part (Table II).

The second virial coefficient, A_2, is included between $4.74 \cdot 10^{-4}$ and $1.09 \cdot 10^{-4}\ cm^3\ mol\ g^{-2}$, proving that DMF is a good solvent for this kind of polymer. From light-scattering data, molecular weights of polymer P103, synthesized in chloroform at a molar concentration of 0.404 mol L^{-1}, and of polymer P110, obtained for a monomer concentration in methanol of 0.391 mol L^{-1}, are, respectively, 30,000 and 68,700 g mol^{-1}. These results confirm that polymers obtained in methanol have higher molecular weights than those resulting from polymerizations in chloroform.

From intrinsic viscosity and molecular weight data (as reported in Table II), the α parameter of the Mark–Houwink–Sakurada relationship $[\eta] = KM^{\alpha}$ can be determined. Assuming that the various polymers are not too polydisperse, a plot of log $[\eta]$ vs. log $[\overline{M_w}]$ (Fig. 5) gives a value of $\alpha = 0.76$. This value is indeed very close to that reported for poly(*N*,*N*-dimethyl acrylamide)[11] in the same solvent (α = 0,81). It suggests that these linear polymers would experience some rigidity due to the presence of relatively bulky substituents in DNMA molecules.

Aldehyde Deprotection

For the polymers, to have functional groups capable of reaction with a primary amine, it was necessary to cleave the acetal moieties to the corresponding aldehyde. For this cleavage, several procedures were tested as seen from Table III.

Strong concentrated inorganic acids as used by Zàbransky et al.[7] or a strong organic acid like paratoluenesulfonic acid (APTS) were too harsh and afforded insoluble dark-colored material. The use of an acidic resin (Amberlit IR120) gave a low deprotection yield (entry 4). Treatment at room temperature with pyridinium paratoluenesulfonate (PPTS) did not allow a total deprotection and removing PPTS by washing the redissolved polymer with a basic solution was unsuccessful.

Complete deprotection was achieved by reacting diluted hydrochloric acid in dioxane. The depro-

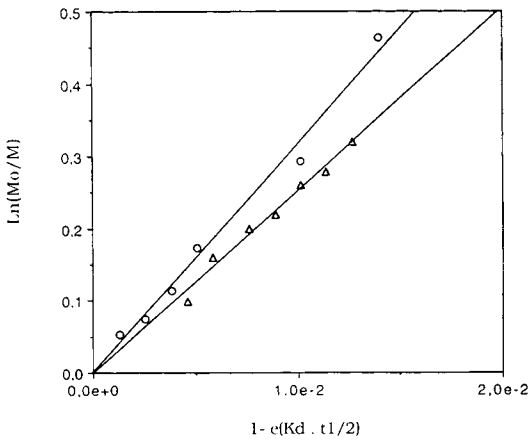

Figure 4 Semilogarithm variation of the monomer consumption, $\ln([M_0]/[M]$ vs. the decomposition rate of initiator: (O) run 1; (△) run 2.

tected polymer which precipitated during the reaction was recovered by filtration, washed with dioxane, and dried under reduced pressure. We observed that the deprotection yield depended on the molar ratio of acid vs. the acetal group (Fig. 6). We also noticed that the water quantity had no influence upon the amount of regenerated aldehyde. So, a simple aldehyde group deprotection method was achieved and, furthermore, the level of deprotection of aldehyde homopolymers could be controlled with the amount of acid involved in the reaction.

Monitoring the aldehyde formation by infrared spectroscopy was not reliable since the 2730 and 1720 cm^{-1} bands could not always be detected. Reagents for the chemical analyses of aldehyde, based on the reaction of a hydrazine derivative[12] with aldehyde moieties, were not appropriate as they react as well with acetal derivatives. In our case, the well-known phenol-based quantification of aldehyde[13,14] gave erratic results. Therefore, the analysis of the formation of aldehyde groups was performed by ^1H- and ^{13}C-NMR, though this latter method is only qualitative. The ratio of the ^1H peak area at 9.4 ppm, corresponding to the aldehydic proton, vs. the peak area of the acetalic proton at 4.5 ppm, afforded the deprotection yield. ^{13}C-NMR was a very convenient tool to make sure that oxidation of aldehyde groups had not occurred on deprotection, as had been noticed by Zàbransky's group.[7] DEPT 135 ^{13}C-NMR experiments on the deprotected polymers confirmed that the carbon atom of the carbonyl group was bound to a hydrogen atom.

Coupling of Oligonucleotides

The purpose of this work was to develop a grafting procedure of oligonucleotides onto the deprotected polymers for use in diagnostics applications. The goal was to tether on the polymeric material as many ODNs as possible with a maximum coupling efficiency so as to reduce the waste of expensive biological material. Two procedures were tested: one in a batch where all the reactants were introduced at once, and the second one being a sequential process.

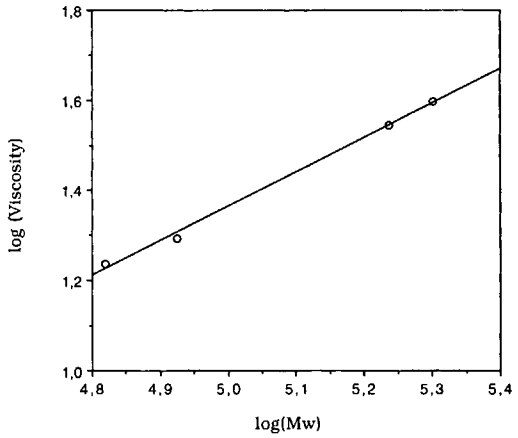

Figure 5 Logarithm plot of the average intrinsic viscosity $[\eta]$ vs. the weight-average molecular weight $\overline{M}_w$.

One-step Procedure

The reaction of a primary amine onto an aldehydic carbonyl group results in the formation of a labile imine bond which has to be stabilized by reduction of the carbon–nitrogen double bond with a borohydride derivative. Due to the very high specificity of sodium cyanoborohydride toward the imine bond, many procedures include this reactant in the coupling medium.[15] Therefore, the aldehyde polymer, the oligonucleotide, and the reducing agent were all together, in differing reaction conditions. In 100% aqueous buffer, no coupling occurred at all, nor as for conditions with mixtures containing 10% of water and 90% of DMSO or DMF.

In acetonitrile and diisopropylethylamine (DIEA)-based mixtures, after a 72 h incubation time at 37°C, depending on the conditions, the coupling yield was up to 29% (Table IV). No improvement of the yield was obtained by increasing the reaction time and,

Table II Molecular Weight Characterization of Poly(DNMA) by Light Scattering

Run Code	Polymer	DNMA (mol L^{-1})	Molecular Weight (g mol^{-1})	dn/dc (mL g^{-1})	A_2 (cm^3 mol g^{-2})	Rg (nm)	Intrinsic Viscosity (mL g^{-1})
3	P103	0.404	30,000	0.072	$4.74 \cdot 10^{-04}$		
7	P107	0.64	109,000		$3.16 \cdot 10^{-04}$	20.4	
8	P108	0.581	66,000	0.072	$1.09 \cdot 10^{-04}$	7	17.3
10	P110	0.391	68,700		$3.05 \cdot 10^{-04}$		
11	P111	0.583	84,000	0.067	$2.91 \cdot 10^{-04}$	10.9	19.6
12	P112	0.792	172,000	0.065	$2.25 \cdot 10^{-04}$	15.2	35
13	P113	1.156	200,000	0.066	$1.94 \cdot 10^{-04}$	19.1	39.5

Table III Homopolymer Deprotection Conditions and Results

Entry	Polymer	Polymer Concentration (g L^{-1})	Acid Concentration (g L^{-1})	Ratio Acid/Acetal (%)	Time (h)	Yield (%)
1	P102	11.1	PPTS 1.8	11	48	30
2	P102	11.1	PPTS 2.6	16	24	30
3	P104	11.0	PPTS 1.8	11	72	24
4	P106	5	IR 120	—	24	10
5	P108	20	HCl 0.1*M*	30	3.5	100

in fact, the results observed after 112 h at 37°C were worse than those after 72 h, since after 112 h at 37°C, water-insoluble aggregates had formed which contained the polymer–ODN conjugate. The formation of these aggregates could be explained by the crosslinking of the oligonucleotide/polymer adducts due to the high amounts of aldehyde groups susceptible to reaction with amino groups on the guanine, adenine, and cytosine bases of the DNA probe.

Reducing the amount of DIEA lowers the yield (runs 1 and 2) but with a lower impact on the course of the reaction than a decreased water content (Table IV, runs 3 vs. 4). With mixtures containing 10% of acetate buffer pH 4.5 and 90% of acetonitrile, no coupling occurred although an acidic medium is well known to activate carbonyl compounds; this can be explained by the protonation of the amine which, therefore, is unable to react as a nucleophile.

Two-step Procedure

Later on, it was found that the reducing agent could be held responsible for the low coupling yields. As shown in Table V, after a 67 h reaction time, the coupling yield reached 49% for homopolymer P109,

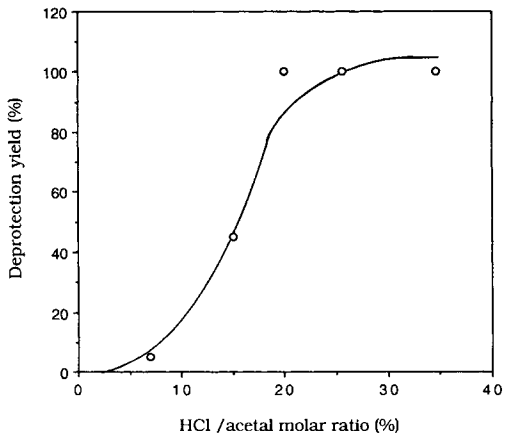

Figure 6 Polymer deprotection yield as a function of hydrochloric acid concentration (run with polymer P107).

whereas in the presence of the reducing agent, a 29% coupling yield was observed in 72 h. But, checked by HPLC 24 h after purification, the stability of the products obtained after coupling without any reducing step was poor. The products stored at +4°C in solution had lost 73% of the "bound" ODN, proving that it was crucial to stabilize the imine bond by reduction.

So, to be effective and allow high coupling yields, the reduction step had to take place after the coupling step had occurred, by adding an aqueous solution of a reducing agent to the reaction mixture. Using this two-step procedure, the coupling yield was improved up to 65% (Table VI). Considering that the formation of the Schiff base is an equilibrated reaction, our results show that with poly(DNMA) the equilibrium must be highly in favor of the formation of the imine bond. Furthermore, this step must be the determining one in the coupling process as longer reaction times allow higher coupling yields as can be seen from Table V.

The nature of the reducing agent has a striking effect: the use of the cyanoborohydride derivative did not allow any product to be isolated, due to a precipitate formation. One possible explanation might be that, by reducing selectively the imine bond, this reagent displaces the equilibrated reaction of amine groups onto aldehyde groups of the polymer toward the production of the Schiff base. This dis-

Table IV Coupling of ODN (Sequence A) with Polymer P109

Run	DIEA (μL)	Water (μL)	Coupling Yield (%)
1	40	28	29
2	20	28	18
3	50	12	11
4	50	3	2

150 μg of the deprotected homopolymer in chloroform, coupling time 72 h, acetonitrile 890 μL; $NaBH_3CN$: 64 μg/mL. CHO/NH_2 molar ratio = 22. ODN concentration: 5 μ*M*.

Table V Coupling Conditions and Results Without Reducing Agent

Run	Polymer	Water (μL)	Time (h)	Coupling Yield (%)
1	P109[a]	16	19/67	16/49
2	P114	16	19	26
3	P115	24	19/67	18/55

Acetonitrile: 890 μL; DIEA 20 μL; polymer: 150 μg. P114 and P115 obtained as P109, but the monomer concentration was, respectively, 0.228 and 0.101 mol L^{-1}. ODN concentration: 5 μ*M*, sequence A.

placement would result in an increase in the cross-linking of ODN molecules with aldehyde polymer molecules to such an extent that it would entail precipitation of the adduct. This precipitation phenomenon is not observed with sodium borohydride, which actually allows the best coupling yields. A reason for this could be the fact that sodium borohydride, being less specific than its cyano counterpart, can undergo several secondary reactions. First, the reduction of aldehyde species to hydroxylated derivatives can occur, consuming the remaining carbonyls groups on the polymer and preventing, therefore, any further reactions. Second, hydrolysis of the hydride to hydrogene, in the aqueous media, consumes the sodium borohydride, preventing extended reduction of imine groups.

A series of coupling reactions were run to assess the role of various factors on the course of the grafting reaction. The polymer and oligodeoxynucleotide concentrations as well as the DNA fragment base composition, the so-called sequence, were examined.

Polymer Concentration

The yield of coupling ODN to polymers increases with the polymer concentration (runs 6 and 7, sequence A, and 13, 14, and 15, sequence B).

Oligonucleotide Concentration

The effect of the variation of the ODN concentration on the formation of water-soluble polymer–ODN conjugates seemed to be sequence-dependent:

With sequence A, a higher DNA fragment concentration reduced the immobilization yield (entries 1, 2 and 12, 13). In the case of run 12, the ODN concentration was too high, and a large amount of insoluble precipitate was obtained. A decrease in the ODN concentration (entry 13) prevented precipitation and the observed coupling yield was up to 39%.

With sequence B oligonucleotides, the opposite effect was observed and increasing the probe concentration resulted in a better coupling yield (runs 5 and 6).

Nevertheless, increasing the initial ODN concentration is a means of increasing, despite reduced yields, the number of DNA fragment molecules bound to one polymer chain.

ODN Fragment Composition

The most striking effect on the immobilization yield was due to the DNA fragment sequence. As a general trend, the yields were better with sequence A ODN probes rather than with sequence B oligodeoxynucleotides (compare entries 4 and 5 in Table VII). The low yields in trying to bind covalently sequence B ODN to polyaldehyde homopolymers were in accordance with the results obtained involving polymers bearing either *N*-hydroxysuccinimide active esters or maleic anhydride groups.[16] These results help to understand the differing effects of the ODN concentration on the coupling yields observed above. As sequence B ODN are poorly reactive, the reaction is dependent on the amount of the DNA probe in the coupling mixture and increasing the concentration displaces the reaction toward the formation of imine bonds, according to Le Chatelier's law. For sequence A ODN that gives high coupling yields,

Table VI Effect of the Reducing Step on the Coupling Yield

Polymer	Polymer Concentration (mg mL^{-1})	ODN Concentration (nmol mL^{-1})	Coupling Yield (%)		
			No Reducing Agent	$NaBH_4$	$NaBH_3CN$
P101	0.15	15	62	65	0
P103	0.15	15	42	45	0

Solvent: water 52 μL; acetonitrile 890 μL; DIEA 20 μL. ODN sequence: A.

the reactivity of the oligonucleotide is not a limiting factor for the reaction to occur. Therefore, increasing the ODN concentration may not increase the coupling yield.

Entries 9, 10, and 11 show interesting results, pointing out the role of the ODN chain length. A 20 base poly(dT) gave a 35% yield in entry 10, although when a 13 base poly(dT) was put to reaction with the same polymer P103, a surprising 0% yield was observed. The same 0% yield was found with a 32 base poly(dT) onto the polymer P107.

All these results suggest that the conformation of the oligonucleotide plays an important role in the course of the reaction. For a yet unclear reason, on increasing the DNA probe length or changing its base composition, the 5′ primary amine is sometimes not available for coupling and probably is embedded in a conformation arising from intramolecular interactions, such as hydrogen bonding for instance, between the different bases of the considered DNA synthetic fragment.

Polymer/ODN Conjugate Characterization

The products resulting from the grafting process were purified by size-exclusion chromatography. The HPLC traces (one is displayed in Fig. 7) show that the polymer–ODN conjugates are excluded in the void volume, which tends to demonstrate that the molecular weights after coupling are over 500 kD (exclusion limit of the column). This very high value

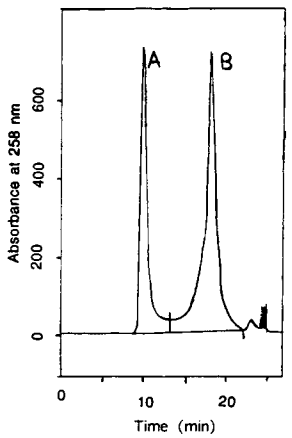

Figure 7 HPLC purification profile of run 13. pic A: conjugate ODN/polymer; pic B: unbound ODN.

in molecular weight can only be explained by the crosslinking of several polymer molecules, bearing DNA probes, due to either the reaction of amino groups of the A, G, and C bases of the DNA molecules, with aldehyde groups of the polymer, as demonstrated in run 12, Table 7, where too high a concentration in DNA probe resulted in the formation of a precipitate, or the intermolecular condensation of aldehyde groups. This could be an explanation for the result obtained in entry 10, Table VII: a 35% coupling yield was observed with a 20 mer poly(dT) nucleotide, which bears no reactive amino group on the thymine bases and which should therefore only react with the aldehyde groups of the polymers by

Table VII Coupling Results of ODN onto Aldehyde Homopolymers

Entry	Polymer	Polymer Concentration (mg mL^{-1})	ODN Concentration (nmol mL^{-1})	CHO/NH_2 Molar Ratio	Time (h)	Coupling Yield (%)	ODN Sequence
1	P105	0.14	42	N.D	96	32	A
2	P105	0.15	15	N.D	43	47	A
3	P101	0.15	15	N.D	120	45	A
4	P103	0.15	12	4.8	120	65	A
5	P103	0.15	15	6	96	9	B
6	P103	0.15	45	19	90	25	B
7	P103	0.075	45	9.5	90	15	B
8	P103	0.04	90	0.8	90	1.6	B
9	P103	0.15	15	6	90	0	C
10	P103	0.15	15	6	90	35	D
11	P107	0.27	79	N.D	66	0	E
12	P109	0.26	157	10	66	9	A
13	P109	0.26	79	20	66	39	A
14	P109	0.13	79	10	66	34	A
15	P109	0.07	79	5	66	20	A

See ODN sequences in Experimental part.

the amino group at its 5′ position with no ability to form crosslinked species. Size-exclusion chromatography of this product displayed the same excluded peak pattern as shown in Figure 7 for a crosslinked ODN–polymer conjugate, proving that self-condensation of aldehyde groups could be held responsible for the crosslinking.

Another hypothesis for the formation of high molecular weight structures during coupling of ODN to linear water-soluble polyaldehyde homopolymers is to consider the possible formation of nonspecific hydrogen bonds between DNA probe molecules. The binding of the probes onto the polymeric synthetic macromolecules would stabilize by a proximity effect secondary nonspecific interactions between the ODN molecules, which are not observed in solution, resulting in the formation of the observed high molecular weight structures; nevertheless, this cannot apply for the result in entry 10 as poly(dT) cannot associate. Finally, these associations require a high salt concentration to occur, which is not the case in this study. This issue is still under investigation in the laboratory.

CONCLUSIONS

Radical-initiated homopolymerization of *N*-(2,2 dimethoxyethyl)-*N*-methyl acrylamide either in methanol or chloroform allowed us to obtain functional polymers which, after deprotection of the aldehyde moiety, were capable of binding nucleic acid probes for diagnostic applications. The recovery of aldehyde groups can occur under mild conditions, and depending on the experimental procedure, the percentage of free aldehyde ranges from 0 to 100%.

The coupling of oligonucleotides requires a reduction step to stabilize the adduct, taking place after the formation of the Schiff bases. The yields of the coupling steps are dependent on the reactant concentrations, mainly the polymer concentration, and the DNA probe sequence.

More work is currently in progress in the lab on several aspects: (i) to select experimental conditions allowing a better control of the yield and molecular weights of DNMA homopolymers; (ii) to complete the characterization of these polymers in the coupling medium conditions so as to obtain a more precise insight of their physicochemical properties in dilute solutions; (iii) to improve the coupling of DNA probes and further characterize the conjugate structures by reliable techniques; and (iv) to study the consequences of these structures on the performances of the conjugates in the considered diagnostic applications.

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