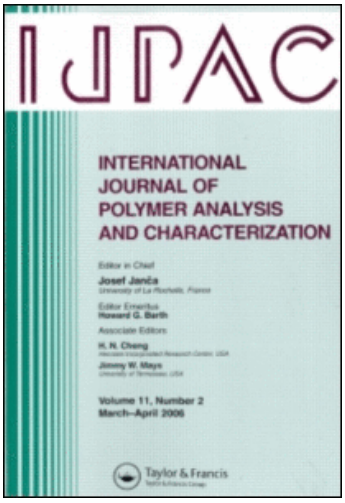

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Characterization of the Grafting Reaction Between Oligodeoxyribonucleotides and N-Vinyl Pyrrolidone /N-Acryloxy Succinimide Copolymers by Size Exclusion Chromatography and Free Solution Capillary Electrophoresis

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The grafting reaction of oligodeoxyribonucleotides (ODN) on N-vinyl pyrrolidone (NVP)/N-acryloxy succinimide (NAS) copolymers has been investigated. First, NVP/NAS copolymers were prepared by free-radical polymerization in N,N-dimethylformamide (DMF); characterization of the monomer sequence distribution by ^{13}C NMR suggested a strongly alternating structure. Low-conversion copolymers were synthesized using azeotropic composition (60% molar NAS) and various overall monomer concentrations. These model copolymers were characterized by light scattering and viscosity measurements in a DMF-water (90/10 volume ratio) mixture.

The covalent attachment of ODN (constituted of 20 bases terminated with a primary amine linker) onto one selected copolymer (60% molar NAS) was then examined. Size exclusion chromatography (*SEC*) and free solution capillary electrophoresis (FSCE) were performed in order to analyse the reaction products and to calculate the yield of grafting. After optimization of the immobilization conditions, the influence of buffer pH and temperature on the grafting reaction kinetics was considered.

KEY WORDS Oligodeoxyribonucleotides, N-vinyl pyrrolidone/N-acryloxy succinimide copolymers, covalent binding, size exclusion chromatography, free solution capillary electrophoresis

INTRODUCTION

The polymerization and the chemistry of activated esters have been recently developed to provide a versatile and simple route to functional polymer synthesis. This method has been

found quite suitable for producing functional copolymers to be used in many domains: chemistry, engineering, biotechnology and medicine [1]. In the biomedical field, copolymers with activated esters of unsaturated acids are particularly well-adapted for reacting with biomolecules containing primary amino groups. In recent work, copolymers of N-isopropyl acrylamide and N-acryloxy succinimide were used for the immobilization of various types of biomolecules: monoclonal antibodies [2,3] immunoglobulins IgG[4], and enzymes[5]. For selective inhibition of gene expression by antisense oligodeoxyribonucleotides, poly (L-lysine) has been coupled to oligonucleotides thus favouring the delivery of antisense sequences to intact cells[6]. Oligodeoxyribonucleotides conjugated to polyvinyl amine or polyacrylic acid have been claimed to increase the sensitivity for nucleic acid hybridization in diagnostic applications[7]. However, in these works, only the biological properties of the corresponding bioconjugates were considered, but no systematic study of the grafting step between oligodeoxyribonucleotides and polymers has been reported.

N-acryloxy succinimide (NAS)-N-vinyl pyrrolidone (NVP) solution copolymers have been selected as model supports since NVP as comonomer should provide some interesting features such as hydrophilicity and biocompatibility[8,9]. The copolymerization of N-vinyl pyrrolidone with N-acryloxy succinimide has been recently reported by Nazarova et al.[10]. Kinetics of this copolymerization and more precise characterization in terms of composition and monomer sequence distribution have been published elsewhere[11]. Some of the main and important results will be summarized in this work.

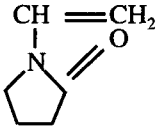
The objective of this paper is to describe the grafting reaction of oligodeoxyribonucleotides (ODN) onto well characterized NVP-NAS copolymers.

EXPERIMENTAL

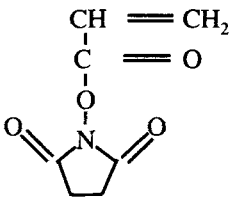
Preparation of N-vinyl Pyrrolidone and N-acryloxy Succinimide Copolymers

N-vinyl pyrrolidone (from Aldrich) was purified by reduced pressure distillation. Commercial N-acryloxy succinimide (from Kodak) was recrystallized in a pentane/ethyl acetate mixture; it was also synthesized from N-hydroxy succinimide and acryloyl chloride according to the method described by Pollak[5].

Copolymerization procedure Solution copolymerization of NVP and NAS has been investigated at 60°C in N,N-dimethyl formamide (DMF) with 4,4′ azobis (4-cyano pentanoic acid) as initiator. The entire procedure has been described in a previous paper[11]. After synthesis, the copolymers were precipitated at low conversion with ethyl ether, filtered, washed several times with the same solvent and finally dried under vacuum.



a: NVP



b: NAS

Synthesis of Oligodeoxyribonucleotide

The oligodeoxyribonucleotide used in this study was synthesized on an ABI 394 instrument (Applied Biosystems, Foster City, USA) using standard cyanoethyl-N,N diisopropyl amino-phosphoramidite chemistry. A 5′-end modification was obtained using the phosphoramidite derivative of the trifluoroacetylaminohexanol (Aminolink 2 from Applied Biosystems). This substitution, after total deprotection, led to a primary amino group, which allows us to perform specific reactions of grafting. After a deprotection step with aqueous ammonia solution, the crude product was precipitated by addition of 3 M sodium acetate and cold ethanol (–20°C) and then isolated in the sodium salt form. Analytical checks were done by ion exchange HPLC using a Gen Pak™ Fax column (Waters) employing a gradient of 20–40% 1 M NaCl in 25 mM Tris HCl buffer pH = 8, over 30 minutes. The purity of ODN, as determined by HPLC was greater than 80%.

Its quantification was performed by measuring the UV absorption (A_{260}) at 260 nm using the following equation:

$$c = \frac{A_{260}100}{(1.54n_A + 0.75n_C + 1.17n_G + 0.92n_T)} \quad (1)$$

Where c is the concentration (nmole/mL) of the oligodeoxyribonucleotide at 260 nm, n_A, n_C, n_G, n_T the number of nucleotides A, C, G, T respectively present in the ODN. The ODN sequence which was used in this work is: $^{5'}$ XTCATCCACCTGGCATTGGAC $^{3'}$ where X = hexamethyleneamine linker

Copolymer Characterization

Activated esters composition UV spectrometry was found to be an appropriate method to determine the activated ester composition. The principle was based on the analysis of the absorption band of the N-hydroxy succinimide (NHS) anion resulting from the aminolysis of the esters by NH_4OH. This spectrometric assay has already been described by Miron et al. [12] for the study of N-hydroxy succinimide esters. The method was found reliable for the determination of the activated esters composition.

The following procedure was used: 25 mg of the copolymer were first dissolved in 10 mL of DMF. After complete dissolution of the copolymer, 10 mL NH_4OH 0.1 M and 80 mL of water were added and the mixture was agitated at room temperature for two hours. 20 µL of this solution were diluted with 380 µL of the mixture (DMF 10%, NH_4OH 0.1 M 10%, Water 80%). The optical density of this dilution was then measured at 260 nm on a Uvikon 901 spectrophotometer (Kontron, Zurich, Switzerland). Under these conditions, the molar extinction coefficient was determined as $\varepsilon_{NHS,\ 260\ nm}$ = 7100 L mole^{-1} cm^{-1}

Copolymer hydrolysis kinetics A similar procedure can be applied to follow the copolymer hydrolysis kinetics. In a 1 mL content quartz cuve, 50 µL of a copolymer solution at 1 mg/mL in DMF were added to a mixture of 60 µL of sodium borate buffer 0.1 M pH = 9.3 and 490 µL of DMF. A timer was switched on as soon as the copolymer solution was added. The hydrolysis reaction was performed at 37°C in the spectrophotometer. The maximum absorption wavelength was shifted from 260 to 270 nm, because of the higher DMF content. The optical density at 270 nm was then measured: in these conditions, another value for ε_{NHS} was determined such as $\varepsilon_{NHS,\ 270\ nm}$ = 6700 L mole^{-1} cm^{-1}

Copolymer microstructure High resolution liquid ^{13}C NMR spectroscopy was carried out with a Brucker AC 200 unit working at 50.3 MHz for ^{13}C. Deuterated DMF (d7 DMF) was used as solvent with dioxane as internal standard.

Molecular weight and viscosity studies Molecular weights were determined by light scattering measurements at 22°C in a DMF-water (90/10 volume ratio) mixture, with a Brookhaven instrument equipped with a 2 watt laser model 2560 (Spectra-Physics) (ionized argon). The sample cell was immersed into a decalin bath. The specific refractive index increment (dn/dc) where n is the refractive index and c the polymer concentration was determined by differential refractometer (with a Brice-Phoenix instrument operating at a wavelength of 632 nm) in the same solvent mixture and yielded dn/dc = 0.095 ± 0.0025 mL/g. Measurements were carried at 22°C after dialysis equilibrium in order to account for preferential solvation. Light scattering data were exploited so as to calculate the weight average molecular weight (M_w) and the second virial coefficient (A_2).

Viscosity studies were investigated at 20°C in the same conditions using a capillary viscosimeter (Viscosimatic VCD, Amtec, France).

Grafting of ODN with NVP/NAS Copolymers

First, the immobilization conditions were optimized for various experimental conditions, using the following grafting procedure for the experiments: 15 10^{-9} moles of dried ODN were dissolved in 25 µL of an aqueous buffer, then 200 µL of an organic solvent miscible with water were added. 25 µL of a copolymer solution at 1 mg/mL in the previous organic solvent were mixed to the ODN solution.

The influence of reaction time and temperature was studied; after the reaction, solvent was removed by evaporation under vacuum.

Analysis of the grafting reaction The grafting reaction was followed using two experimental techniques: size exclusion chromatography (SEC) and free solution capillary electrophoresis (FSCE).

SEC A Kontron device (Zurich, Switzerland) was used equipped with an ultrahydrogel column (UH 500, Waters 30 cm × 7.6 mm), with porosity 5 10^2 or 10^3 Å. The eluent was a sodium phosphate buffer 0.1M pH 6.8 at a flow rate of 0.5 mL/min, and detection was performed by measurement of the optical density at 260 nm.

Purification of the Copolymer/ODN Conjugate

Copolymer-ODN conjugates were purified by SEC, using the above mentioned conditions. The purified conjugates in the sodium borate buffer (2 mL) were dialysed against distilled water (1 L) at room temperature during two hours or at 4°C during 12 hours. The ODN concentration of the purified conjugate solution was determined by UV spectrophotometry at 260 nm.

FSCE Capillary electrophoresis was performed using the 270 A-HT system (Applied Biosystems, Foster City, USA) equipped with a silica capillary of 72 cm length and 50 µm diameter. The capillary was thermostated at 40°C. Samples were introduced into the capillary by an electrokinetic method for 1.5 s. The separation was performed in a sodium

carbonate buffer (0.05 M; pH 9.6) at 20 kV. U.V detection was used at a 260 nm wavelength. Before each analysis, the capillary was rinsed with 0.1N NaOH for 5 min and then equilibrated with the buffer for 10 min.

Grafting Reaction: Kinetic Studies

Grafting kinetics were followed by SEC or by FSCE under the same experimental conditions than for the copolymer hydrolysis study. The grafting protocol has been slightly modified taking into account the results of the optimization experiments. The influence of pH and temperature has been thoroughly investigated.

Ten Eppendorf vials containing 15.10^{-9} moles of dried ODN were prepared. For each vial: ODN was dissolved in 30 µL of 0.1 M sodium borate buffer at pH depending on the experiment and then, 245 µL of DMF were added. 25 µL of a copolymer solution at 1 mg/mL in DMF were mixed in the ODN solution. The mixture was incubated with agitation at a temperature depending on the experiment. The reaction was stopped by adding 50 µL of 4 10^{-3} M NH_4OH solution, in order to hydrolyse rapidly the N-hydroxy succinimide esters. Solvents were removed by evaporation under vacuum. The dried reaction product was then redissolved in 50 µL of deionized water and analysed.

RESULTS AND DISCUSSION

Preparation of the copolymers

Based on a previous study[11], it was found that the NVP-NAS radical-initiated copolymerization exhibited a set of reactivity ratios such as:

$$\text{NVP} = 0.01 \pm 0.02$$
$$\text{NAS} = 0.30 \pm 0.05$$

These values were expected from the literature which gives reactivity ratio data for many binary systems containing NVP[10,13,14]. The reactivity ratio related to NVP is indeed always near zero whereas that of NAS (as well as for various ester acrylates) is generally much larger. Since the product $r_{NVP} \cdot r_{NAS}$ tends towards zero, it is clear that a strong alternating tendency is present in the copolymer chains. In order to avoid compositional heterogeneity according to the set of reactivity ratios, it was decided to synthesize copolymers at the azeotropic composition ca. 60% molar of NAS, which is favorable to get homogeneous copolymers. In addition, copolymerizations were stopped at relatively low conversion so as to avoid broad molecular weight distributions.

Copolymer Characterization

Copolymers synthesized at the azeotropic comonomer composition were characterized with regard to microstructure and molecular weight.

Copolymer microstructure For a better description of the copolymers, it was attempted to determine the monomer sequence distribution by NMR spectrometry. A splitting of the NVP (V) carbonyl resonance was observed. This was ascribed to a compositional effect

together with a simultaneous configurational effect brought about the NAS unit (A). On the contrary, no compositional effect was observed for the NAS. As shown in Figure 1, the enlargement of N-vinyl pyrrolidone carbonyl resonances for a copolymer prepared at azeotropic composition exhibits four peaks, with the absence of any VVV long sequences. If only compositional effect was considered, three rays should have been observed corresponding to A*V*A, A*V*V and V*V*V triads, respectively. Because of configuration effect induced by A unit, five peaks were expected (mm, rm + mr and rr of A*V*A, *r* and *m* of A*V*V). mm, rm+rm, and rr are respectively isotactic, heterotactic and syndiotactic triads.

Table 1 compares the monomer sequence distribution for two samples of a copolymer prepared at the azeotropic monomer composition, and precipitated at 10% and 95% conversion. It is clear that this copolymer is preferentially alternating (main contribution of isolated units) with a fairly homogeneous composition. This could be expected from the simulation of the triad distribution, when considering a terminal kinetic model; the pres-

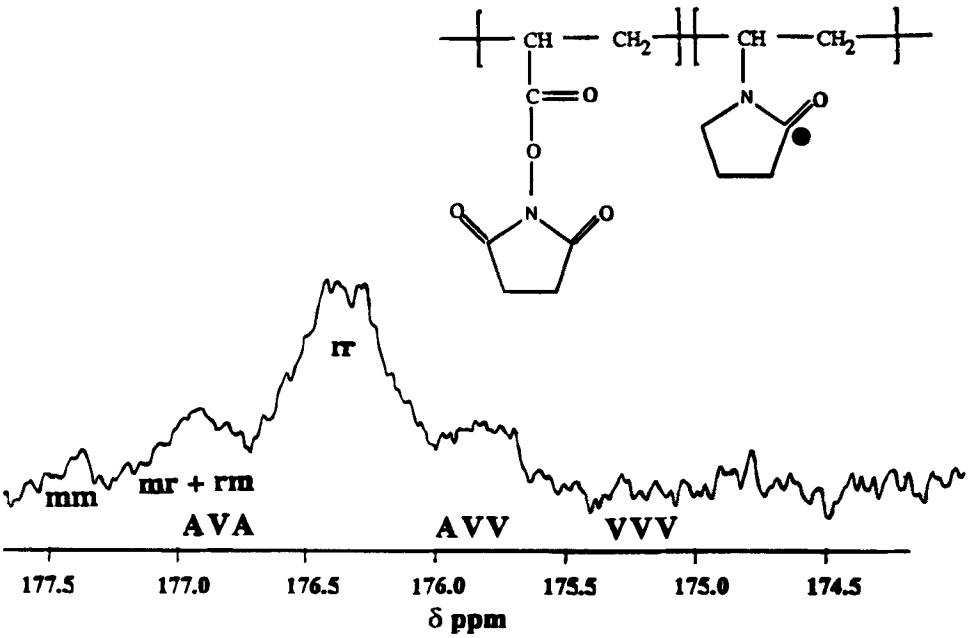


FIGURE 1 ^{13}C NMR spectrum (50.3 MHz) of a copolymer around the azeotropic composition (64 % molar of NAS); enlargement of the N-vinyl pyrrolidone carbonyl resonances.

TABLE I

Average copolymer composition and average-NVP centered triads distribution determined by UV spectrometry and ^{13}C NMR respectively.

	Conversion (%)	Copolymer composition mol % NAS +/– 5%	A*V*A (%) +/– 5%	A*V*V (%) +/– 5%	V*V*V (%) +/– 5%
AZEO1.1	10	55	82	18	0
AZEO1.4	95	65	88	12	0

ence of AVA and AVV triads is only shown. Moreover, at the azeotropic composition, the copolymer is homogeneous independent of the conversion, and the distribution of the comonomer units along the polymer chain is invariant.

Molecular weights and viscosity studies Molecular weights and intrinsic viscosities were determined from light scattering and viscosity measurements at 20°C in a DMF-water (90/10 volume ratio) mixture since the dn/dc value was negligibly small in pure DMF.

From intrinsic viscosity and molecular weight data, it was possible to estimate the α parameter of the Mark-Houwink-Sakurada relationship using the formula: $[\eta] = K\,Mw^{\alpha}$. The plot of log[η] versus log Mw (Figure 2) provided a value of the α parameter = 0.5; if one takes into account that this plot was established with polydisperse copolymer samples (even they were prepared at low conversion), this α value would indicate that the chain exhibits a random coil conformation. As reported in Table II, copolymers exhibit molecular weight ranging between 19500 and 135000 g/mole. In addition, the second virial coefficient is also given, with an average value (2. 10^{-4} cm^3 mole/g^2) indicative that the

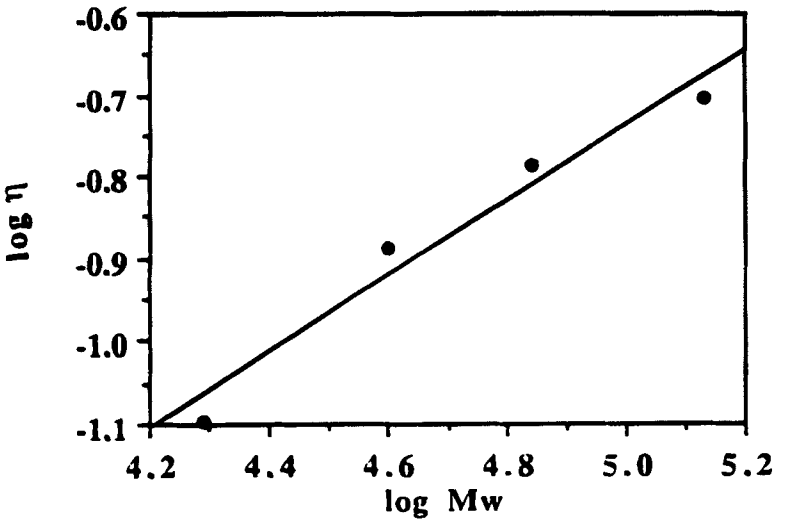


FIGURE 2 Determination of the α parameter of the Mark-Houwink law, in a DMF-water (90/10 volume ratio) mixture.

TABLE II

Characterization of copolymers at azeotropic composition. Molecular weights and viscosity measurements, at 20°C in a DMF-water (90/10 volume ratio) mixture.

	Average copolymer composition (mole % NAS)	$\overline{M}_w$ g/mole	Second virial coefficient: $A_2 \times 10^4$ cm^3 mole /g^2	[η] dL/g
AZEO9	61	19500	2.02	0.080
AZEO7	60	40000	2.04	0.130
AZEO1	62	69000	2.35	0.163
AZEO2	61	90000	1.98	0.176
AZEO5	61	135000	1.32	0.197

selected solvent mixture behaves as a relatively good solvent for this copolymer, regardless of the M_w.

Hydrolytic stability (UV spectrometry) Copolymer hydrolysis kinetics were studied under similar conditions as for grafting reactions one for the sake of comparison. However, it was not possible to follow the hydrolysis of the activated esters with the ODN, since the maximum absorption wavelengths of N-hydroxy succinimide and ODN are very close. The copolymer AZEO1 was used for this study.

Four different borate buffers 0.1 M, pH 7.5, 8, 8.5 and 9.3 were tested. It was assumed that the molar extinction coefficient ε determined for N-hydroxy succinimide in the DMF-borate 0.1 M pH = 9.3 (90/10 volume ratio) mixture did not change in the case of other pH solutions. The experimental procedure has been previously described[11]. The slope of -Ln(a) versus time where a is the number of NHS groups on the copolymer (in moles), considering the linear part of the curve, is equal to the hydrolysis rate constant (Figure 3, Table III). As showed in Figure 3, first order kinetics could be observed during the first five minutes, thereafter the reaction's order decreases. The copolymer is relatively stable

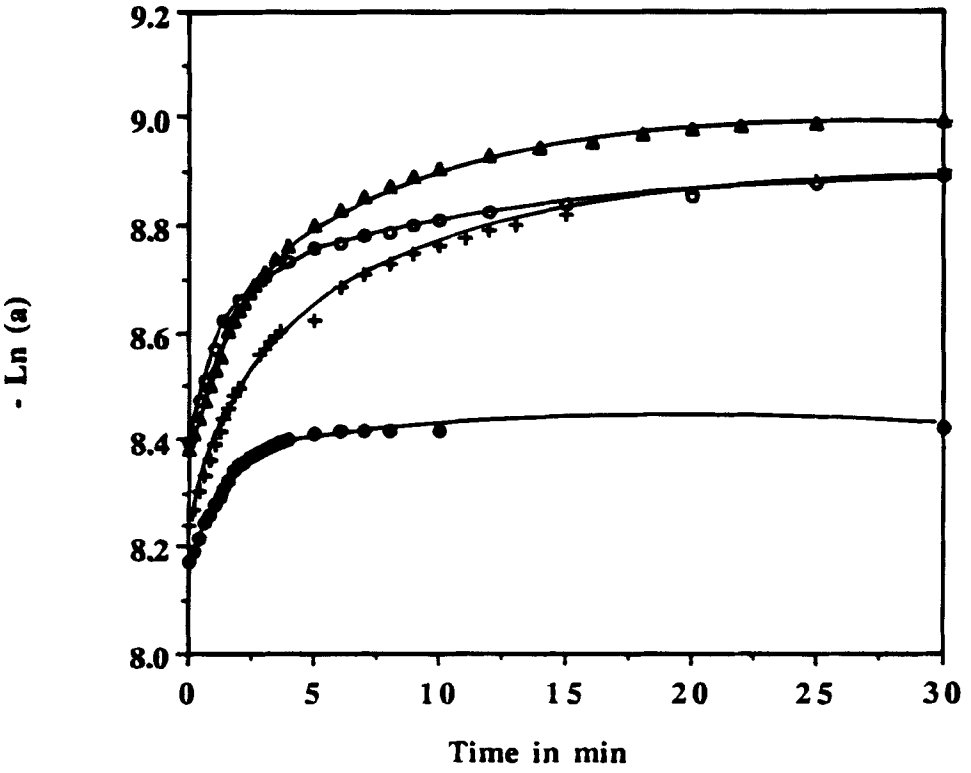


FIGURE 3 Influence of the buffer pH on the hydrolysis kinetics.
-Ln(a) pH = 7.5 ●
-Ln(a) pH = 8.0 +
-Ln(a) pH = 8.5 ▲
-Ln(a) pH = 9.3 ○

TABLE III

Hydrolysis rate constants for different borate buffers pH and hydrolysis yield after 60 minutes of reaction at 37°C.

Borate buffer pH	Hydrolysis rate constant (s^{-1}) 10^3	Hydrolysis yield after 60 minutes of reaction (%)
7.5	1.70	30
8.0	2.30	55
8.5	2.60	60
9.3	3.30	60

towards hydrolysis at neutral pH. However, as the pH is increased from 7.5 to 9.3, hydrolysis kinetics become faster. In Table III, the maximum hydrolysis yield as a function of the pH is reported. Surprisingly, hydrolysis reactions were not complete although a very large excess of water with respect to the esters was used. Before starting the reaction, as previously indicated, copolymer chains exhibit an expanded random coil conformation in the grafting medium. The rapid hydrolysis of the first esters favours the chain expansion because of repulsive interactions induced by carboxylate charges; then as more charges are incorporated in a high DMF content medium, the copolymer chains would collapse in a more compressed coil structure which decreases the accessibility of the activated esters.

Grafting of ODN to the Copolymer

The reaction was carried out in a water/organic mixture since the copolymer sample is only soluble in polar solvents (DMF, DMSO or N-methyl pyrrolidone), whereas the ODN is water-soluble. Two competitive reactions should be actually considered: hydrolysis and grafting as schematically described in Figure 4.

SEC and FSCE analysis Two analyses are depicted in Figure 5 for the grafting of ODN on the copolymer AZEO1: the first is by FSCE and the second by SEC according to experimental conditions described in the experimental part. It was assumed that:

1 There is no adsorption of the copolymer-ODN conjugate or the free ODN during the analysis. In the case of FSCE analysis, this assumption is probably correct, since an empty capillary was used.
2 The absorbance at 260 nm of the copolymer-ODN conjugate is only due to the oligonucleotides.
3 The molar extinction coefficient of the ODN is the same, whether it is free or covalently bound to the polymer.

The grafting yield (R) can be calculated (with respect to ODN) from the copolymer-ODN conjugate and the free ODN peak areas according to the equation:

$$R(\%) = \frac{(\text{copolymer} - \text{ODN}) \text{ area}}{(\text{copolymer} - \text{ODN}) \text{ area} + (\text{free ODN}) \text{ area}} \cdot 100 \qquad (2)$$

Both methods seem to be in good agreement as illustrated in Table IV for the previous example. By SEC, the copolymer-ODN conjugate and the free ODN are well-separated

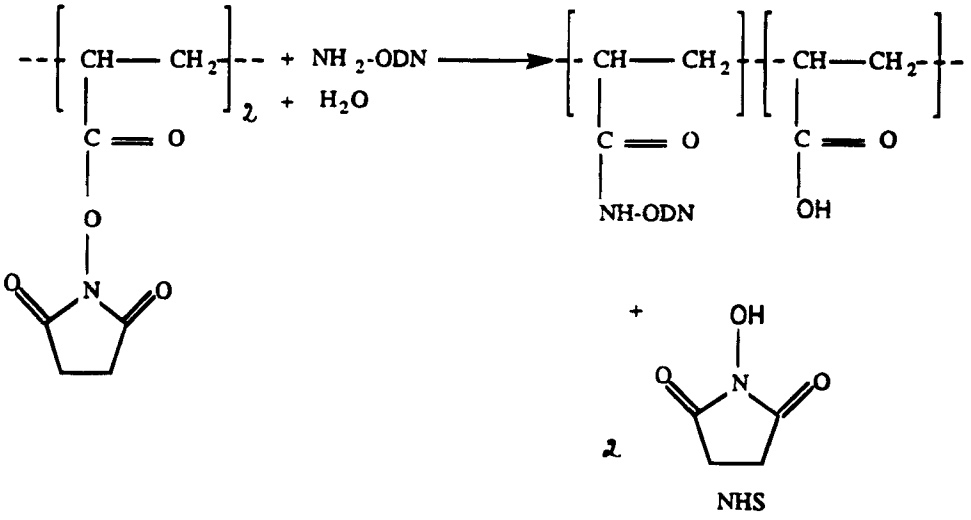


FIGURE 4 Activated esters hydrolysis and ODN grafting reactions.

since they exhibit large differences in molecular weights and consequently various hydrodynamic volumes. Free ODN and ODN-grafted copolymers display different charge densities which make possible their separation by FSCE. It should be mentioned the presence of more than one peak for the ODN which corresponds to lower size ODN molecules; however, due to the nature of the synthesis protocol, only the ODN with the selected length was terminated with the aminolinker. Hence, the determination of the grafting yield was not affected. Both methods provide the same information, however, some differences can be emphasized between the two techniques. On one hand, SEC can be performed for the purification of the copolymer-ODN conjugate which is not feasible by FSCE; on the other hand, analyses are more easily carried out by FSCE than by SEC, and in addition, they are more rapid.

Moreover, the grafting yield has been checked by a fluorescence method [15] which consisted in covalently attaching a fluorophore probe (fluorescein hydrazide) onto NVP-NAS copolymers before performing the ODN grafting. After purification of the conjugate, the copolymer and the ODN were independently quantified using fluorescence and UV spectroscopy, respectively. The grafting yield was deduced and good agreement was observed with the values already given by SEC and FSCE analyses.

Optimization of the Coupling Conditions

First, the immobilization conditions were optimized with a view to controlling the yield of grafting by SEC or FSCE as well as determining the influence of pertinent parameters upon various experimental conditions.

The ODN/activated esters stoechiometry was 1 mole of ODN for 6 moles of activated esters for all the optimization experiments. This ratio was selected as a compromise between two requirements: i) to immobilize a large number of ODN per chain for further use of these conjugates; ii) to take into account that the N-hydroxysuccinimide are close

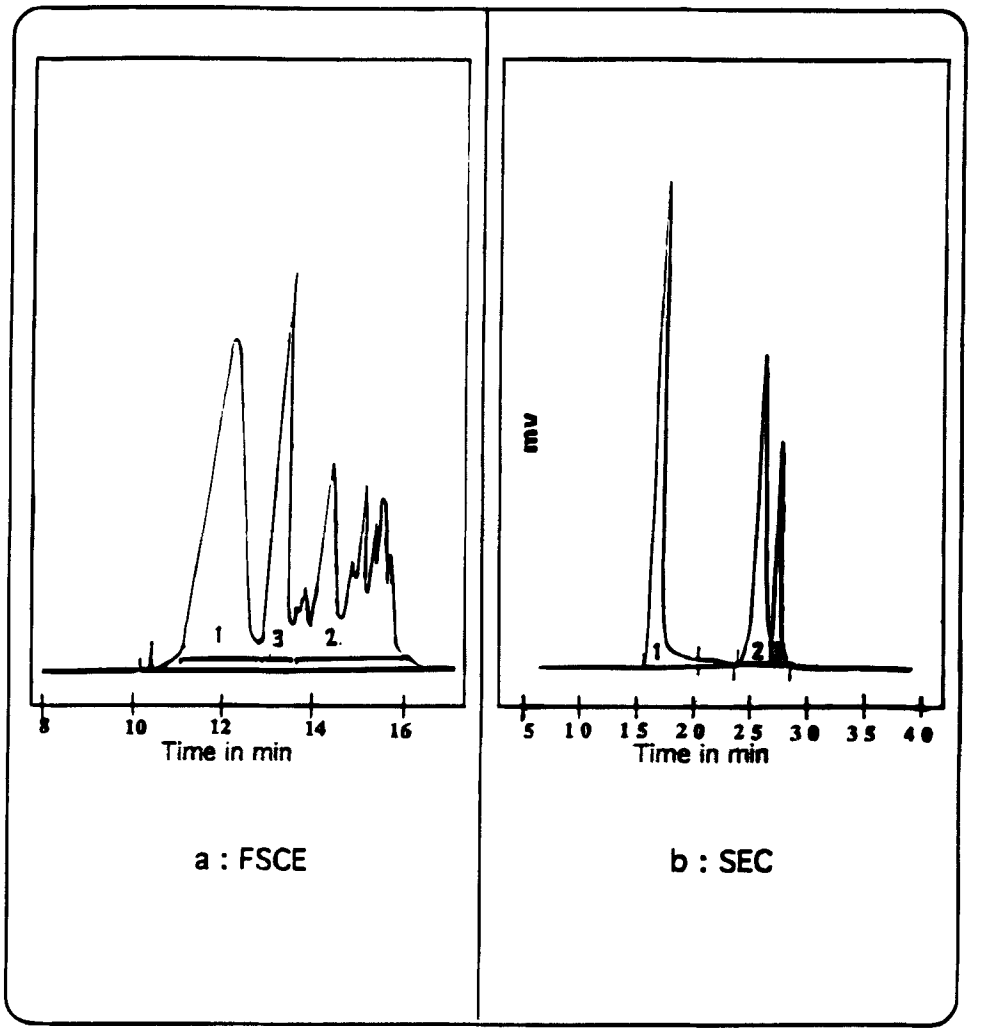


FIGURE 5 Analyses of the grafting reaction of ODN on the copolymer AZEO1 by free solution capillary electrophoresis (a) and size exclusion chromatography (b) 1 = copolymer/ODN conjugate, 2 = free ODN, 3 = NHS

TABLE IV

Comparative of analysis of the grafting reaction by free solution capillary electrophoresis (FSCE) and size exclusion chromatography (SEC) (from Figure 4)

	relative areas (FSCE) (+/–3%)	relative areas (SEC) (+/–3%)
Copolymer/ODN conjugate	54	53
Free ODN	34	36
NHS	12	11
Grafting yield (%)	61	59

to each other, because of the copolymer alternating structure, thereby limiting the grafting reaction by steric hindrance.

The influence of a large number of parameters was investigated using FSCE and SEC as analytical techniques: the nature of the organic solvent, the nature, pH and ionic strength of the buffer, the organic solvent/aqueous buffer ratio, the temperature, the reaction time. DMF and sodium borate buffer were selected because higher coupling yields were reached. No significant effect of the reaction time after one hour was observed since grafting reaction was found to be very fast.

The influence of the aqueous to organic phase volume ratio (W/O) was quite significant. Various (W/O) ratios ranging between 5 and 20% were tested keeping constant the ODN and copolymer concentrations. With 20% of water, the yield of grafting drastically decreases (6% instead of 45%), probably because hydrolysis becomes the predominant reaction. On the contrary, with 5% of water, grafting yield becomes low, (30% instead of 45%), due to the poor solubility of ODN at high DMF contents. The influence of the temperature and of the buffer pH has been studied and the results are reported in the next section.

Kinetic studies

For a better understanding of the ODN grafting reaction on the NVP/NAS copolymer, kinetic studies were investigated using both SEC and FSCE as analytical techniques. For all experiments, the copolymer was sample AZEO1.

Effect of the pH. ODN grafting kinetics were performed under the same conditions used than for the copolymer hydrolysis kinetic studies previously described. Four different buffer pH were tested 7.5, 8, 8.5 and 9.3 respectively, keeping constant the concentration (0.1 M) and the temperature (37°C). No grafting reaction was observed for pH 7.5 and pH 8 probably because the amino group of the ODN being partly protonated, is less reactive. Grafting kinetics for pH 8.5 and 9.3 are reported in Figure 6, showing no significant difference except that the reaction is a little faster at pH 9.3. However, the maximum grafting yield is approximately the same at both pH-values, ca. 40% with respect to ODN. Hydrolysis kinetics performed under the same conditions and in the absence of ODN show that 40% of the activated esters are still present in the polymer. Kinetic curves were then interpreted considering a first order law during the first five minutes. The slope of -Ln(a) versus time (Figure 7) where a is the number of free ODN in moles considering the linear part of the curve allow the determination of the grafting rate constants as reported in Table V.

Influence of the temperature Kinetics were followed at four different temperatures 22, 30, 37 and 50°C (Figure 8) by FSCE keeping the pH constant (9.3). From the curves given in Figure 8, there is no significant effect of the temperature on the maximum grafting yield which reaches a value ranging between 40 and 50% for all cases. Grafting rate constants were calculated from the slope of -Ln(a) versus time where a is the number of free ODN (in moles) considering first order kinetics and using the linear part of the curves (Figure 9).

The knowledge of k at various reaction temperatures (T) allows one to apply the Arrhenius equation : $k = k_0 \exp(-E/RT)$ where k is the rate constant in s^{-1}, E the activation energy in joules/mole, R the perfect gas constant and T the temperature in Kelvin degree. A semi logarithm of k versus 1/T was plotted from data of Table VI and a linear variation

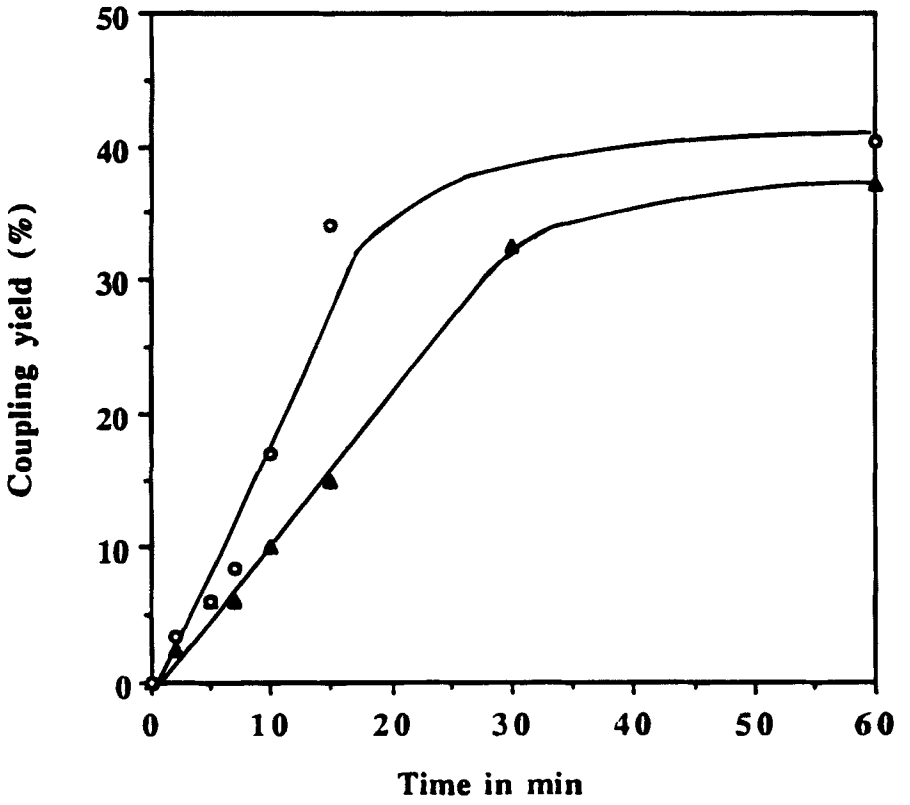


FIGURE 6 Influence of the pH of the borate buffer on the yield of grafting.
–Ln(a) pH = 8.5 ▲
–Ln(a) pH = 9.3 O

was shown. From the slope (-E/R) and Ln k intercept, the activation energy (E) and constant k_o were determined as: E = 80 kjoules/mole and $k_0 = 2.510^{10}$

These experiments on the grafting of ODN onto NVP-NAS copolymers showed the drastic influence of pH and temperature on the kinetics; however in every case, the grafting yield always reached a plateau at ca. 40%. Several reasons might be responsible for such a result: steric hindrance, electrostatic interactions, and conformation changes. It may be expected that steric hindrance would increase as the number of covalent bound ODN onto copolymer is increased despite the fact that a low [ODN]/[activated ester group] ratio was used. Electrostatic interactions could also interfere with the development of repulsive forces acting between the charges created on the copolymers (due to hydrolysis and ODN fixation) and free ODN in solution.

Finally, the conformation of the ODN-copolymer conjugate should also play a predominant role on the accessibility of free ODN. It may be postulated that this conformation would change as the grafting and hydrolysis reactions are proceeding. Based on their solution behavior, it was suggested that the initial copolymer chains exhibited a random coil conformation; then, due to the hydrolysis and grafting reactions, the formed grafts would tend to expand the conformation of these chains (presence of ionic charges and

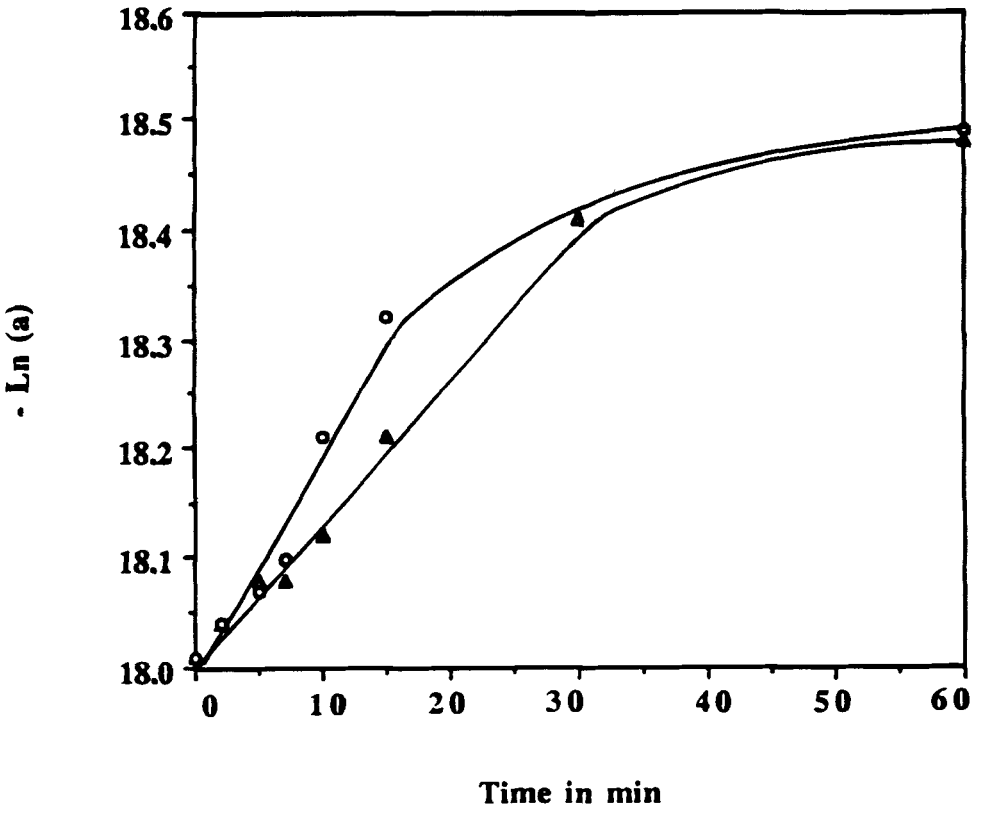


FIGURE 7 Influence of the borate buffer pH on the grafting rate constant.
-Ln(a) pH = 8.5 ▲
-Ln(a) pH = 9.3 ○

TABLE V

Determination of the grafting rate constant and of the maximum grafting yield at different borate buffer pH.

Borate buffer pH	Grafting rate constant s^{-1} 10^4	Maximum coupling yield FSCE analysis (%)
8.5	2.3	43
9.3	3.6	42

ODN). However, as the ionic charges increase, the resulting grafts would experience poor solvent conditions (in a rich-DMF coupling mixture) which could cause the formation of a more compact structure; depending on their location, this would reduce the accessibility of the unreacted activated esters. This very important point is currently being investigated through the study of the physicochemical properties of these ODN-copolymer conjugates in dilute solution.

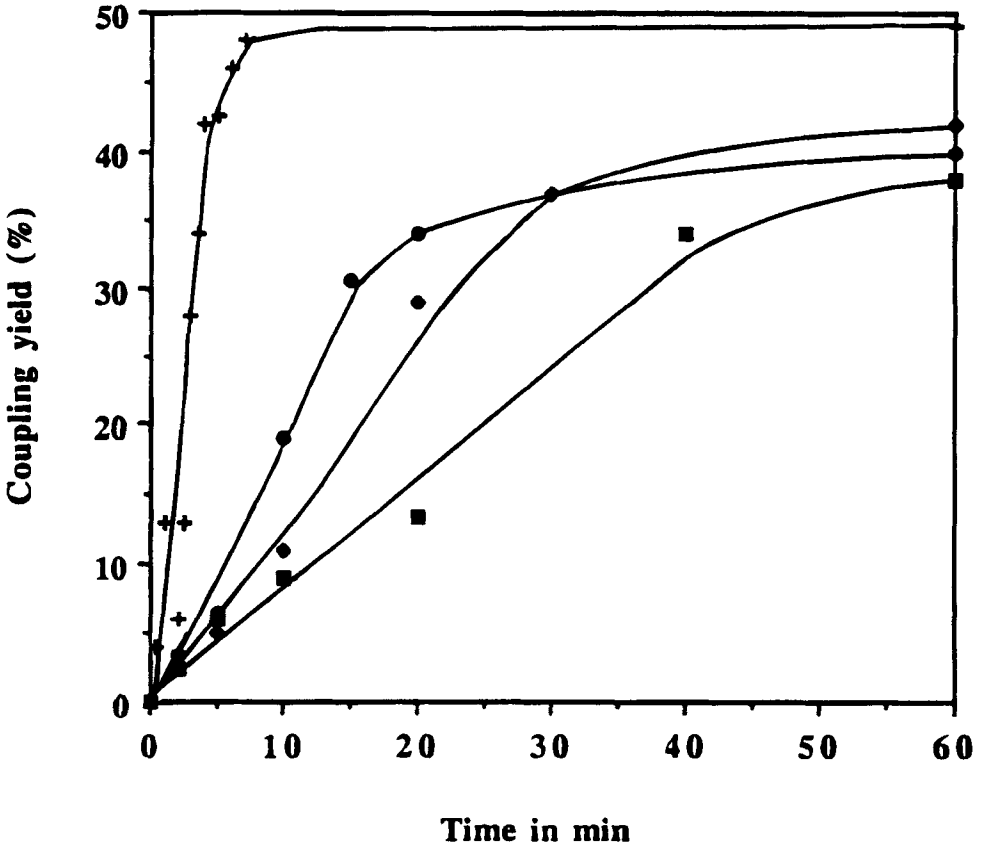


FIGURE 8 Influence of the temperature on the kinetics of grafting. Grafting yield versus time for T = 22, 30, 37 and 50°C

22°C ■
30°C ◆
37°C ●
50°C +

CONCLUSIONS

The covalent immobilization of oligodeoxynucleotides to a N-vinyl pyrrolidone/N-acryloxy succinimide copolymer was investigated. Considering the set of reactivity ratios for this binary system (0.3 for NAS and 0.01 for NVP), it was decided to synthesize copolymers at the azeotropic monomer composition ca. 60% mol of NAS, which is favorable to get homogeneous copolymers. Low-conversion copolymers were prepared so as to avoid broad molecular weight distributions. They were fully characterized with respect to monomer sequence distribution by ^{13}C NMR and to physicochemical properties in a DMF-water (90/10 volume ratio) mixture by light scattering and viscosity studies. This allowed to select a model copolymer for the grafting study of ODN. At first, copolymer hydrolysis kinetics were followed by UV spectrometry in a DMF-borate buffer 0.1 M (90/10 volume ratio) mixture for various pH. The copolymer was found to be relatively

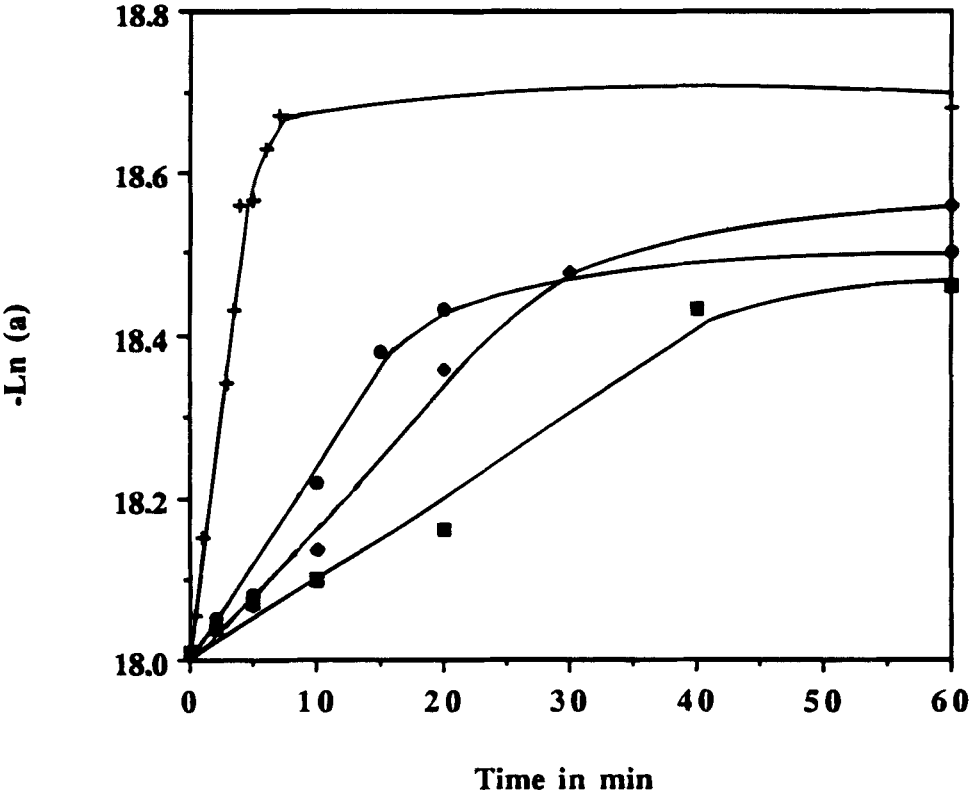


FIGURE 9 Determination of the grafting rate constants for various reaction temperatures: 20, 30, 37 and 50°C.
–Ln(a) 22°C ■
–Ln(a) 30°C ●
–Ln(a) 37°C ●
–Ln(a) 50°C +

TABLE VI

Influence of the temperature on the kinetics of grafting: determination of the coupling rate constant and of the activation energy.

T(°C)	T(°K)	$1/T$ (K^{-1}) 10^3	k (s^{-1}) 10^4	Ln k (s^{-1})
22	295	3.38	1.3	–8.98
30	303	3.30	2.7	–8.22
37	310	3.22	3.6	–7.95
50	323	3.09	2.3	–6.06

stable at neutral pH, but hydrolysis kinetics were faster for higher pH. The grafting of ODN onto the copolymer was then examined using FSCE and SEC. Both techniques allowed us to determine the yield of grafting with respect to ODN and, in addition, reaction kinetics were followed. These reactions were found very fast but incomplete (maximum grafting yield near 50%) for steric hindrance reasons and because of the copolymer conformational change as the reaction proceeds, causing a decrease in the accessibility of the activated esters. A more detailed analysis was performed on the effect of pH and tem-

perature, showing that the pH value was quite critical; the reaction grafting drastically decreased at pH below 8 and was overbalanced by hydrolysis above pH 10.

The structure of these copolymer/ODN conjugates are presently being studied further using light scattering and neutron scattering techniques. Moreover, the applications of these conjugates in medical diagnostic tests involving nucleic acids have been developed and will be published later on.

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