Reactive Polymers in Diagnostics: Syntheses and Characterizations of Nucleic Acid Probes and Maleic Anhydride-*co*-Methyl Vinyl Ether Polymers

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ABSTRACT: Maleic anhydride-*co*-methyl vinyl ether copolymers were used as reactive polymers to link oligodeoxyribonucleotides (ODN) to make oligonucleotide–copolymer conjugates of potential applications in diagnostics. The anhydride moieties of the copolymers were used for the covalent binding, via the formation of a peptide bond, on reaction with DNA probes that were amino modified at the 5′ position. The best coupling yields were obtained by carrying out the reaction in a DMSO-borate buffer (95/5 volume ratio) mixture at high ionic strength. Copolymers and ODN–copolymer conjugates were characterized by size exclusion chromatography and multiangle laser light scattering detection. The average molecular weights of the formed conjugates were relatively high, in the 10^6 g/mol range, resulting from the crosslinking of several copolymer molecules during coupling. Coupling DNA probes onto smaller molecular weight polymers did not result in the formation of very high molecular weight conjugates showing that copolymer chain interpenetration was a determining factor of the crosslinking process that occurs during the coupling reaction. © 1997 John Wiley & Sons, Inc. J Appl Polym Sci **65:** 2567–2577, 1997

Key words: maleic anhydride copolymers; light scattering; size exclusion chromatography; oligodeoxyribonucleotides; immobilization

INTRODUCTION

Linear synthetic functional polymers have been shown to be very useful in various diagnostics applications. For instance, they have been bound to proteins and oligonucleotides to increase the coating efficiency of allergens[1] and nucleic acid probes,[2] or to increase the detection signal in various tests.[3]

Maleic anhydride copolymers can be regarded as preactivated polymers due to the presence of the anhydride moieties susceptible to yielding an amide bond on reaction with a primary amine of a biomolecule. Several authors have already used the copolymer anhydride groups to covalently link biomolecules either in the solid state, as a support functionalizing agent,[4] or in solution to prepare insoluble enzyme complexes.[5]

Here we report a study on the syntheses and characterization of soluble conjugates of nucleic acid probes and maleic anhydride-*co*-methyl vinyl ether polymers (PMAMVE) with special attention on the kinetics of the coupling reaction and on the physicochemical characteristics of the resulting conjugates.

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EXPERIMENTAL

Materials

Chemicals

Oligonucleotides were synthesized on an ABI 394 instrument (Applied Biosystems, San Francisco, CA) using standard cyanoethyl-*N*,*N*-diisopropyl aminophosphoramidite chemistry according to the manufacturers' protocols. For the coupling reactions, oligodeoxyribonucleotide (ODN) can be either used as such after cleavage from the resin and precipitation in ethanol, or after purification by HPLC to remove impurities, in particular smaller incomplete ODN. Otherwise the ODN was used unpurified.

Polymer Samples

Alternated copolymers of MA and MVE (PMAMVE) were supplied by Polysciences, Inc. The weight average molecular weights (M_w) of PMAMVE samples 1 and 1A were 67,000 and 20,000 g/mol, respectively.

The detailed physicochemical characterization of the solution behavior of these polymers in various media was investigated and will be published later.[6]

Other chemicals were from Aldrich and were used as received, unless stated otherwise.

Methods

Coupling of Oligonucleotides to Copolymers

The appropriate amounts of polymers were dissolved in anhydrous DMSO at 37°C. DMSO was added to various amounts of oligonucleotides dissolved in various aqueous buffers. To the oligonucleotide (ODN) buffer/DMSO solutions were added the required volumes of polymer/DMSO solutions. After stirring at 37°C, the solvents were removed under reduced pressure prior to any analysis.

Analyses of Coupling Reactions

The coupling yield (R) is defined as the ratio of the amount of polymer bound ODN versus the total amount introduced in the reaction mixture. Crude products were purified by size exclusion chromatography (SEC) using a Waters Ultra-Hydrogel 500 column, a Kontron HPLC 420 pump, and a Kontron 430 UV detector. Purifications were run in a 0.1M phosphate buffer at pH 6.8. Detection was achieved by measuring the absorbance at 260 nm corresponding to the ODN. (At the concentrations used, the polymer had no absorption.) The ratio of the peak area corresponding to the polymer bound oligonucleotide versus the sum of the two peaks corresponding to the unbound and the bound ODN (i.e., the total amount of probe involved in the reaction) gave the coupling yield (R), assuming that the bound and unbound oligonucleotides had the same specific molar extinction coefficients.

SEC-Mulitangle Laser Light Scattering (MALLS) Experiments

These experiments were performed on-line with the following high performance SEC setup.

A Waters Ultra-Hydrogel 500 column (or a Waters Ultra-Hydrogel 500 column associated with a Waters Ultra-Hydrogel 1000 column) and a Waters 510 high performance liquid chromatography pump, running with a 0.1M phosphate buffer (pH 6.8) as eluent and at a flow rate of 0.5 mL/min, was used. For the detection part, a Waters 484 absorbance detector, a Waters 410 differential refractometer, and a three angle MINI DAWN F detector (Wyatt Technology) operating at 690 nm were used on-line.

Kinetics of Coupling Reaction

To follow the course of the coupling of the DNA probes (ODN) onto copolymers, 35-μL aliquots of the reaction mixture were pipetted off and the reaction was quenched by addition of 10 μL of a 30% ammonium hydroxide solution. After solvent elimination under reduced pressure, the crude was analyzed as described above.

RESULTS AND DISCUSSION

Determination of Experimental Conditions for Coupling of Nucleic Acid Probes onto MA-MVE Copolymers

The binding of oligonucleotides onto these polymers relies on the formation of an amide bond between the primary amine at the 5′ position of a synthetic ODN and one carbonyl group of an anhydride moiety. The anhydride acts as an activated ester for the peptide bond formation; the anhydride ring is opened to give rise to a carboxylate ion and to the desired amide (Fig. 1). In standard organic chemistry, this reaction is run in

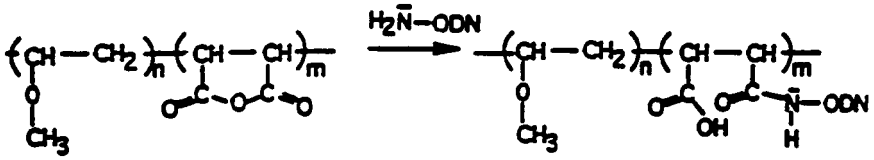


Figure 1 Coupling reaction between ODN and copolymer (PMAMVE).

anhydrous solvents to avoid the side reaction of hydrolysis by eventual water traces. In our case, water was required for the solubility of the oligonucleotide in the reaction mixture; therefore, we had to determine the optimum conditions in which hydrolysis is minimum and coupling maximum. Thus, for the covalent binding of oligonucleotides to MA-MVE copolymers, the following parameters were studied:

1. the nature of the organic solvent;
2. the nature of the aqueous buffer;
3. the use of a catalyst; and
4. the duration of the reaction, the temperature, the pH of the buffers, and the ionic strength.

Results are reported in Table I.

Effect of Nature of Organic Solvent on Course of Reaction

Among the various solvents tested (runs 1–4, Table I), DMSO yielded the best results for the coupling yield. There is still no clear explanation concerning the drastic solvent effect observed in this reaction, but it is worthy to note that differing results were obtained with other polymers: for the grafting of ODN onto *N*-vinyl pyrrolidone *N*-acryloxy succinimide copolymers, DMF appeared to be the best solvent[7]; for the immobilization of oligonucleotides onto poly(*N*-(2,2′-dimethoxyethyl)-*N*-methyl acrylamide), the highest coupling yields were obtained with acetonitrile.[8]

Concerning runs 1, 5, and 6 in Table I; the kinetics of the coupling reaction under the conditions of 4 or 6 h seemed to give the same coupling yield that was somewhat higher than after 2 h of reaction.

Table I Determination of Coupling Conditions on PMAMVE 1 Sample

Run	[ODN] (μmol/L)	Buffer	Buffer (%)	pH	Solvent	Catalyst	Reaction Time (h)	*T* (°C)	Yield (%)
1	48*	Borate 0.1*M*	4	9.4	DMSO		4	37	33
2	48*	Borate 0.1*M*	4	9.4	DMSO/butanol		4	37	18
3	48*	Borate 0.1*M*	4	9.4	Acetonitrile		4	37	1
4	48*	Borate 0.1*M*	4	9.4	DMF	DIEA	4	37	11
5	48*	Borate 0.1*M*	4	9.4	DMSO		2	37	23
6	48*	Borate 0.1*M*	4	9.4	DMSO		6	37	35
7	48*	Borate 0.1*M*	4	9.4	DMSO	DMAP	4	37	33
8	48*	Borate 0.1*M*	4	9.4	DMSO	DIEA	4	37	24
9	48*	Borate 0.1*M*	4	9.4	DMSO	DMAP	4	50	41
10	36**	Borate 0.1*M*	4	9.4	DMSO		6	37	28
11	36**	Borate 0.1*M*	2	9.4	DMSO		6	37	23
12	36**	Tris 50 m*M*	4	9.2	DMSO		6	37	5
13	36**	Carbonate 0.1*M*	4	9.3	DMSO		6	37	7
14	36**	Carbonate 0.1*M,* NaCl 1*M*	4	9.3	DMSO		6	37	54
15	36**	Borate 0.1*M,* NaCl 1*M*	4	9.3	DMSO		6	37	61

Reaction conditions: [PMAMVE 1] = 0.3 g/L; volume, 500 μL. Sequence of ODN used: ODN*, 5′-XAAGGTTTCGGCGGAGATCCT-3′; ODN**, 5′-XGATGAGCTATATGAGAACGGTA-3′ (where X = hexamethylene amine linker).

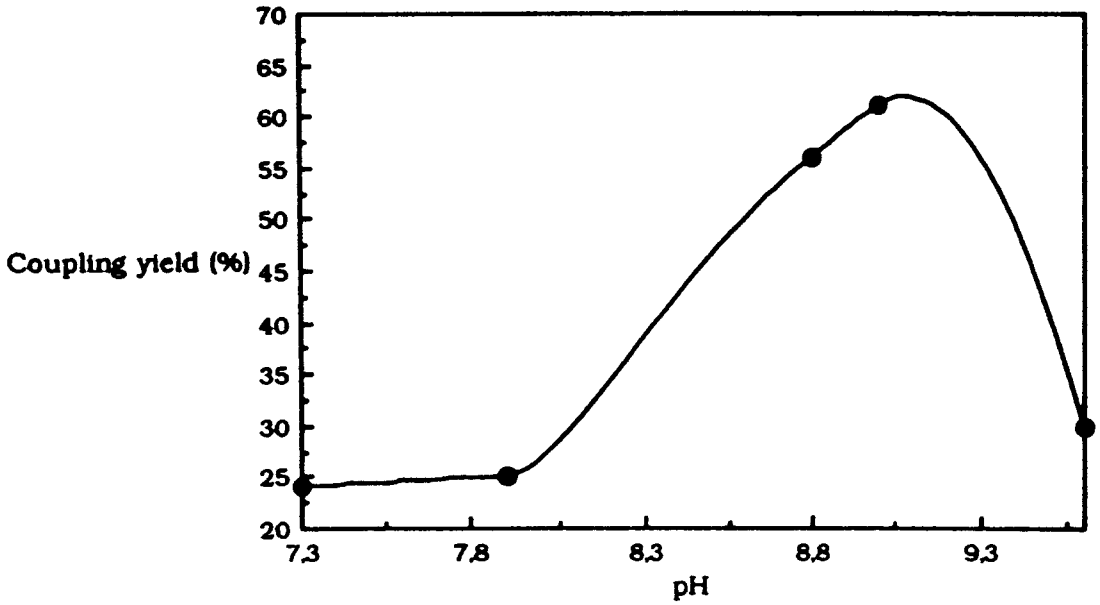


Figure 2 Coupling yield versus buffer pH. Reaction conditions: 5% (0.1*M* borate buffer)/DMSO, 1*M* NaCl, 37°C, 6 h; ODN sequence: 5′-XGATGAGCTATATGAGAA-CGGTA-3′ (where *X* = hexamethylene amine linker).

Known acylation catalysts such as dimethylaminopyridine (DMAP) or diisopropylethylamine (DIEA) turned out to be uneffective at improving the coupling yields (runs 7, 8, and 9) whereas increasing the coupling temperature to 50°C was more efficient (run 9).

Effect of Nature and Ionic Strength of Buffer on Course of Reaction

The role of the buffer was examined first by a twofold reduction of the amounts of borate buffer (run 11, compared to run 10, Table I) without any marked effect. Substituting borate with a Tris buffer (run 12) or a carbonate buffer, (run 13) drastically reduced the coupling yields.

Increasing the ionic strength in a carbonate buffer by adding sodium chloride up to 1*M* (run 14) boosted the reaction from a 7% yield to 54%; using a borate buffer at pH 9, and 1*M* sodium chloride, a 61% yield was achieved. This striking effect due to ionic strength can be explained by taking into account the electrostatic interactions. On alkaline hydrolysis, one anhydride moiety gives rise to two carboxylate anions; at low ionic strength, electrostatic repulsions between these negative charges and the phosphate ions of the ODN prevent an oligonucleotide molecule from approaching a copolymer chain. Adding sodium chloride results in a screening of the charges, allowing the macromolecules to approach one another for the coupling reaction to occur.

Effect of Buffer pH on Course of Reaction

Figure 2 shows the role of the buffer pH on the coupling yield: there is a regular increase of the yield on increasing the pH up to a maximum. Then, it decreases at higher pH values where the hydrolysis kinetics of the anhydride moieties is faster than that of the coupling reaction of the DNA probe onto the polymer. Below pH 8, the grafting reaction is not effective, probably because the partial protonation makes the amino group of the spacer arm at the 5′ position of the ODN less reactive.

From this study on the course of the coupling reaction, the most efficient experimental coupling conditions were 5% of a 0.1*M* sodium borate buffer and 0.5*M* sodium chloride in DMSO at 37°C.

Kinetics of Coupling Reaction and Effect of Polymer Chain Length

Kinetics of Coupling Reaction

With the experimental conditions determined above, we investigated the kinetics of the coupling of oligonucleotides onto MA copolymers. As can

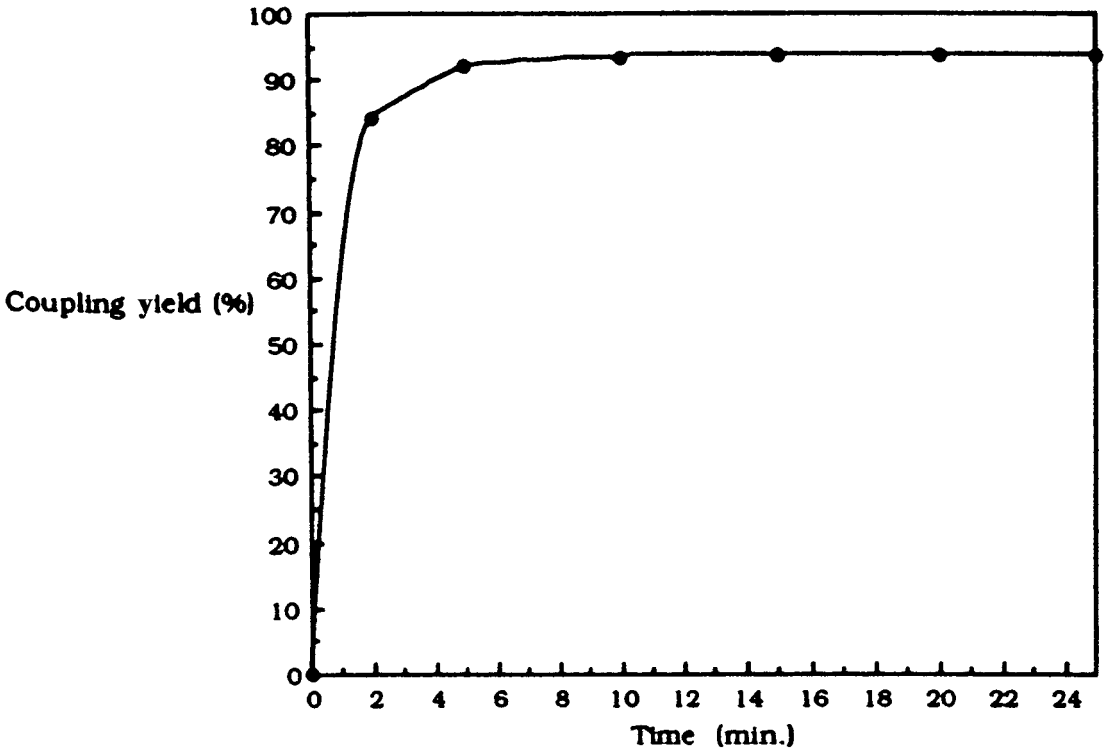


Figure 3 Kinetics of coupling reaction to PMAMVE 1 sample, yield vs. time. Reaction conditions: 5% (0.1*M* borate buffer)/DMSO, 0.5*M* NaCl, 37°C; ODN sequence: 5′-XGAACAGCCAGAAGGAC-3′.

be seen from Figure 3, the immobilization reaction is surprisingly fast; it took place within the first 10 min and reached 93% yield when a purified oligonucleotide was used.

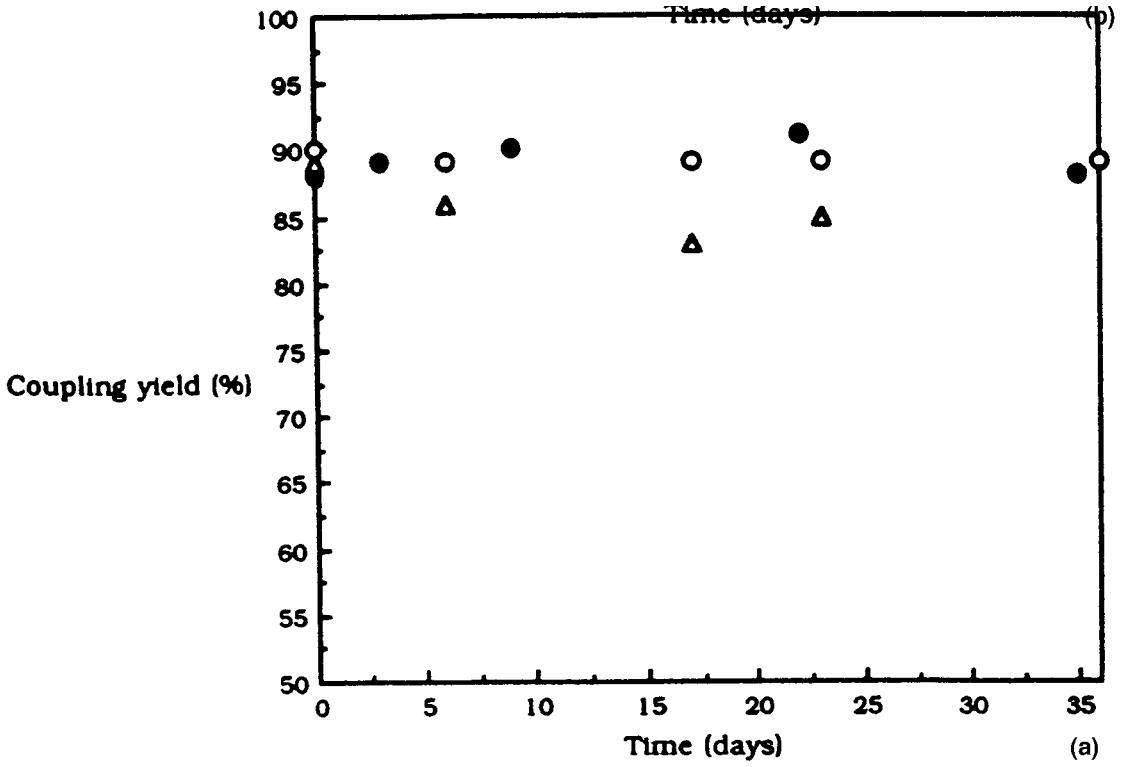
Stability of Coupling Yield

The stability of the conjugates was investigated in water and the coupling medium (5% borate buffer, 95% DMSO) at 4 and 37°C with two polymers of different sizes: PMAMVE 1 and PMAMVE 1A. Figure 4 shows that the conjugates are stable in these solvents and at these temperatures because no decrease in the amount of conjugate is observed with time. So, the bonds involved in the formation of the conjugates are strong and there is probably a covalent bond between the primary amine at the 5′ position of the ODN and one carbonyl group.

It is noteworthy that a non-amino-modified oligonucleotide (sequence: 5′-TCATCCACCTGGCATTGGAC-3′), under standard reaction conditions, reacts with a 10% coupling yield to obtain a conjugate stable with time on storing in water at 4 and 37°C for up to 20 days. This result proves that the amino groups borne by the bases of the ODN are able to react with some anhydride groups of the polymer.

Effect of Polymer Chain Length on Coupling Reaction

The next factor that could have an effect on the course of the reaction was the size of the synthetic polymer involved in the binding reaction of the oligonucleotides. We ran two series of coupling experiments with increasing ODN concentrations using polymer samples PMAMVE 1 and PMAMVE 1A. For comparison purposes, the number of polymer molecules involved in the reaction was kept constant, which meant that for the lower molecular weight polymer, the amount of available reactive groups would be inferior to that of the higher molecular weight polymer. The results reported in Figure 5 show that for both polymer samples, the amount of bound ODN increases with the initial oligonucleotide concentration in the coupling medium to level off at a plateau value of 50 ODN per chain for the PMAMVE 1A sample and 90 ODN molecules for the PMAMVE 1 sample. Assuming oligonucleotides to be covalently bound, these plateau values correspond, respectively, to one ODN



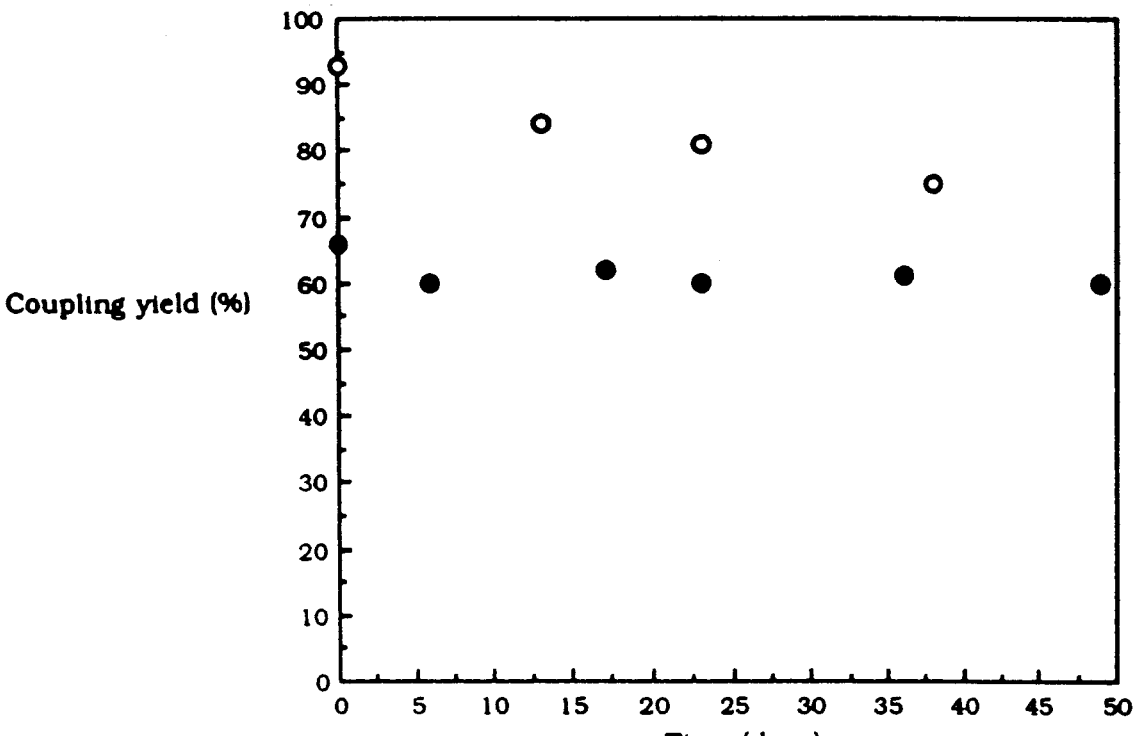


Figure 4 Stability of conjugates at 4 and 37°C in water and in the coupling medium: (a) for PMAMVE 1: (△) in water at 37°C, (○) in water at 4°C, (●) in coupling medium at 37°C; (b) for PMAMVE 1A: (○) in water at 37°C, (●) in water at 4°C.

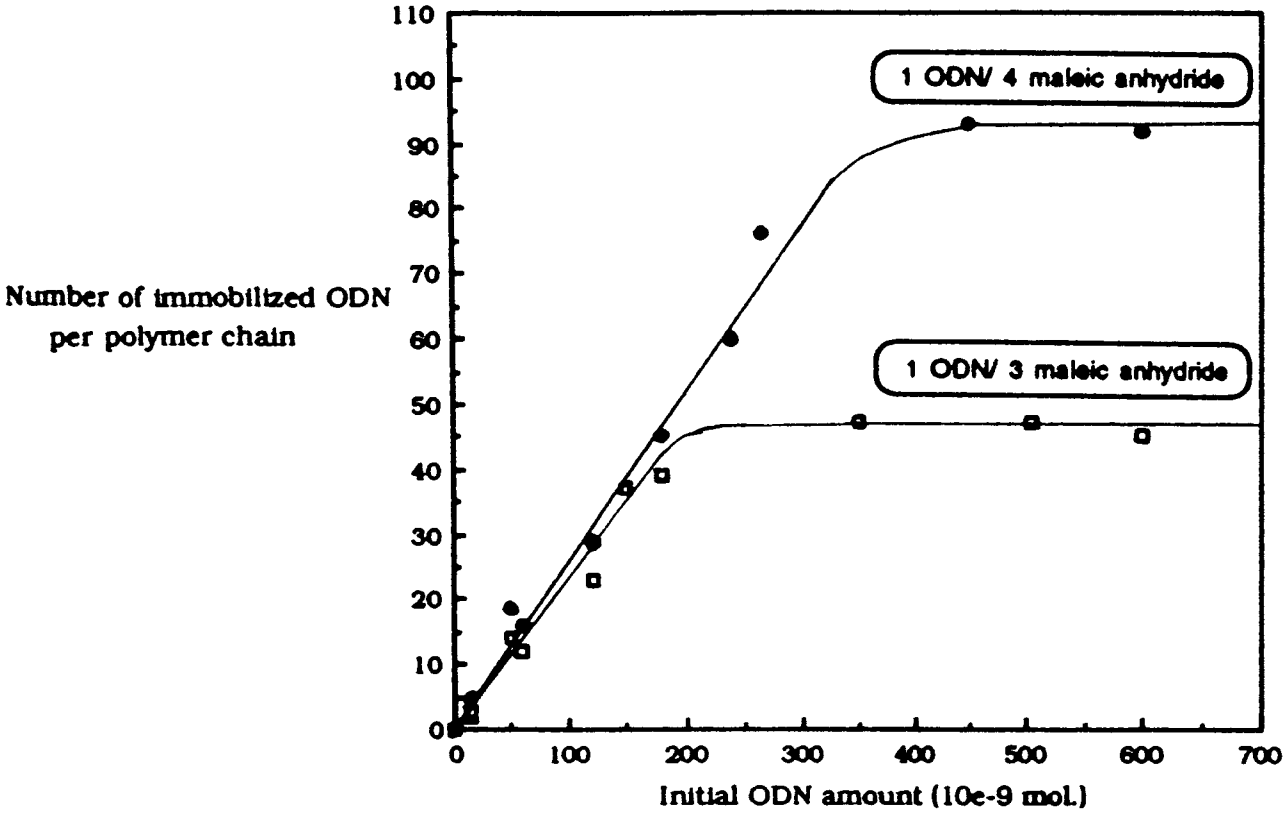


Figure 5 Amount of bound ODN versus the initial number of ODN for (●) PMAMVE 1 and (□) PMAMVE 1A. Coupling reaction: [PMAMVE 1] = 15 g/L, [PMAMVE 1A] = 6 g/L; ODN sequence: 5′-XGTCCTTCTGGCTGTTC-3′.

for three available anhydride moieties and one ODN for four available anhydride moieties for sample PMAMVE 1. These results demonstrate that for longer polymer chains, as in the case of sample PMAMVE 1, the immobilization of DNA probes is inhibited by some sort of steric hindrance. Therefore, on smaller polymer chains, as in the case of polymer PMAMVE 1A, bound oligonucleotides can be more densely packed than on longer ones.

Table II Optical Densities for Various Amounts of ODN with and without Polymer at 260 nm

ODN	Nb ODN Initial (mol)	Absorbance	
		Without PMAMVE	PMAMVE + ODN
1	15E-9	0.4497	0.4347
1	30E-9	0.9254	0.9758
1	60E-9	1.0694	1.0079
2	15E-9	0.5618	0.5507
2	30E-9	1.1846	1.2220
2	60E-9	1.0842	1.1473

ODN sequence: ODN_1, 5′-XGTCCTTCTGGCTGTTC-3′, ODN_2, 5′-XGAGCTGCGTAAG-3′ (with X = hexamethylene amine linker).
Nb: initial ODN amount.

These data were based on the determination of the coupling yield obtained by the method based on the measurement of the absorbance at 260 nm due to the ODN, as described in the Experimental section. To demonstrate that there was no modification of the molar extinction coefficient (ε) on coupling, Table II compares the optical densities measured for various amounts of ODN after reaction with the copolymers with those of samples containing no polymer. For the two different ODNs, the values obtained with or without polymer were identical, indicating that no modification of ε had occurred on coupling to the copolymers.

Physicochemical Analyses of Oligonucleotide–Polymer Conjugates

SEC-MALLS was used as an analytical tool to determine the molecular weight distributions of

Table III Results of SEC-MALLS Analyses for PMAMVE 1/DNA Probe Conjugates

Entry	Conjugate Code	$\bar{M}_{w_{app}}$ (g/mol)	$\bar{M}_{n_{app}}$ (g/mol)	$R_{g_{app}}$ (nm)
1	11	9,453,900	4,801,500	61.3
2	12	3,569,300	2,308,400	62.1
3	3	2,815,800	1,576,400	67.8
4	4	1,788,300	1,286,900	66.6

the copolymer–ODN conjugates (see the Experimental section for more details). Only sample PMAMVE 1 was used and the refractive index increment for the conjugates was kept as 0.2 mL/g, corresponding to the value for DNA.[9] The results reported in Table III show that, on binding with oligonucleotides, very high molecular weight conjugates are obtained with rather compact conformations as shown by the gyration radii of ca. 65 nm. These so-called "aggregates" could result from the crosslinking of several polymer chains during coupling and their formation could explain why two kinds of peaks were observed in the SEC for the polymer–oligonucleotide conjugates (Fig. 6). In this HPLC trace, three major peaks are observed. One at 18 min corresponds to the unreacted oligonucleotide. The other two peaks correspond to the conjugate; one at 10 min is obviously excluded (the exclusion limit of the column according to the manufacturer is 400,000 g/mol), and the second one at 11 min corresponds to lower molecular weight coupling products. This possible crosslinking was already observed with other reactive polymers,[7,8] and we suppose either a chemi-

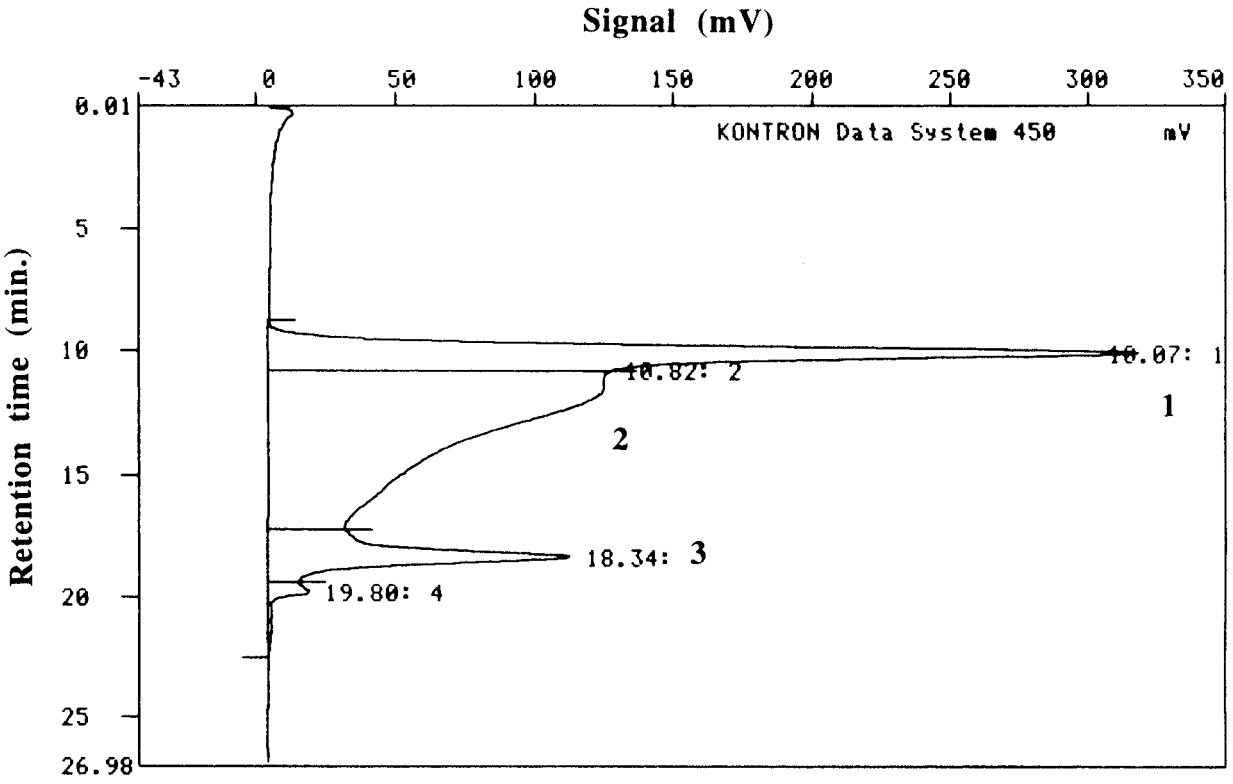


Figure 6 SEC trace of the coupling reaction mixture showing three kinds of peaks corresponding to (1) the excluded peak, (2) lower molecular weight conjugates, (3) unreacted oligonucleotide.

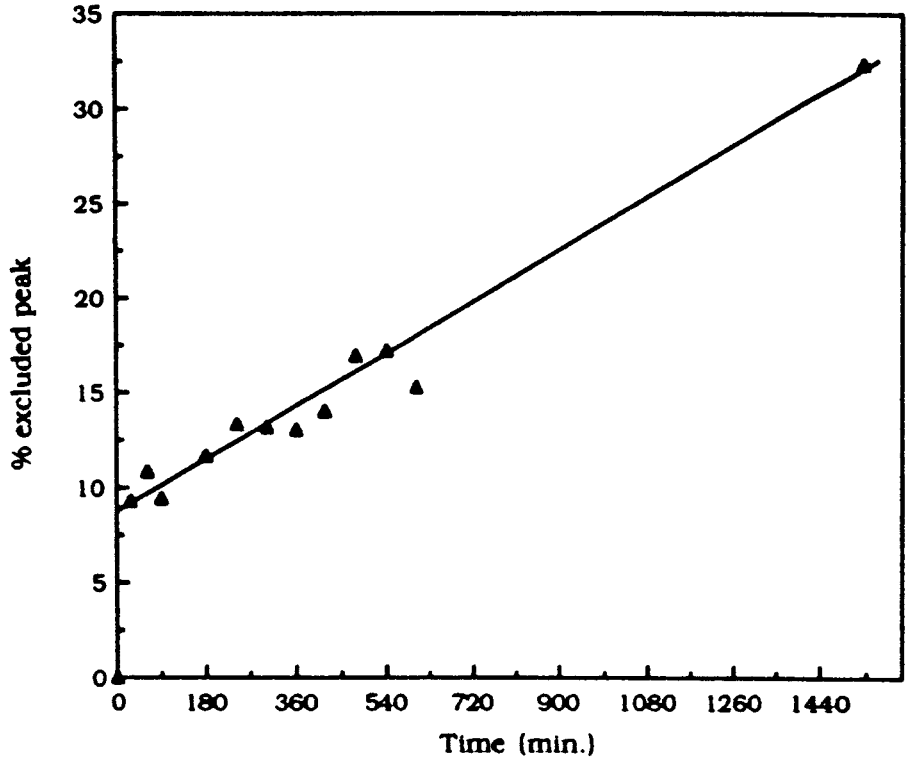


Figure 7 Kinetics of formation of ODN–copolymer molecular associations; percent excluded peak vs. time. ODN sequence: 5′-XGAACAGCCAGAAGGAC-3′.

cal reticulation involving amine moieties of the bases of the ODN with the remaining reactive groups on the polymers or noncovalent interactions between the bound oligonucleotides and the polymer or other bound ODNs occurred.

It is questionable whether or not the aggregate formation might result from the drying process necessary to remove the excess organic solvent prior to the SEC-MALLS analyses. This was checked by injecting a small amount of the crude reaction mixture (consisting mainly of DMSO) into the SEC column. The corresponding HPLC trace was exactly identical to that obtained after injection of a sample dried under reduced pressure (i.e., treated according to the protocol described in the Experimental section).

To determine the nature of the interactions responsible for the occurrence of the aggregates, we studied the kinetics of their formation as well as their stability in various media and under different conditions.

Kinetics of Aggregate Formation

We defined percent excluded peak as the ratio of the area of the excluded peak versus the sum of the areas of the excluded and nonexcluded peaks, corresponding to the amount of ODN–copolymer conjugate actually formed during coupling. This ratio was monitored as a function of time and the results are reported in Figure 7. The figure shows that the aggregate formation is a slow process that proceeds after the coupling reaction reaches completion. Indeed, Figure 3 shows that the maximum coupling yield is obtained in less than 10 min.

Stability of Aggregates

Several experiments were run to assess the stability of these aggregates when stored, by studying the evolution of the ratio (percent excluded peak) over long periods of time.

In the coupling medium (5% borate buffer, 95% DMSO) the conjugates were stored at 37°C; the amount of the excluded peak, corresponding to the aggregate, increased until day 3 and then was stable up to day 45 after the experiment ended (Fig. 8).

The product was stored in water after elimination of the organic solvent under reduced pressure. In water, the aggregates were less stable

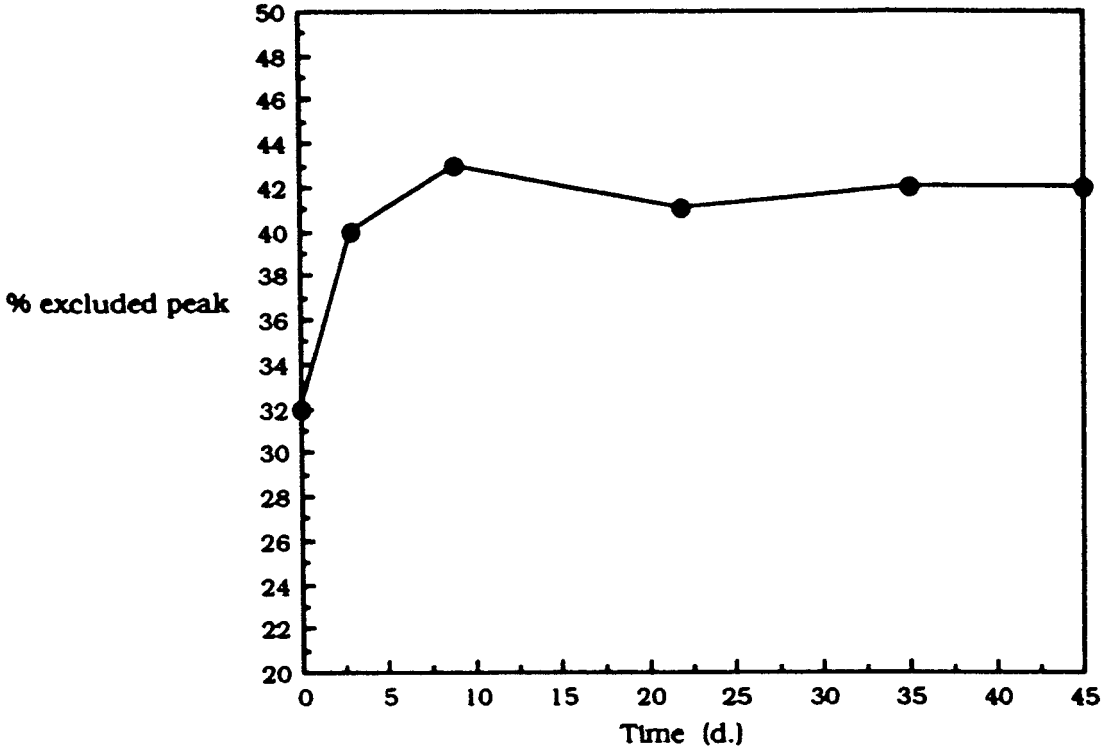


Figure 8 Stability of excluded peak of aggregates in coupling medium (DMSO) at 37°C. ODN sequence: 5′-XGTCCTTCTGGCTGTTC-3′.

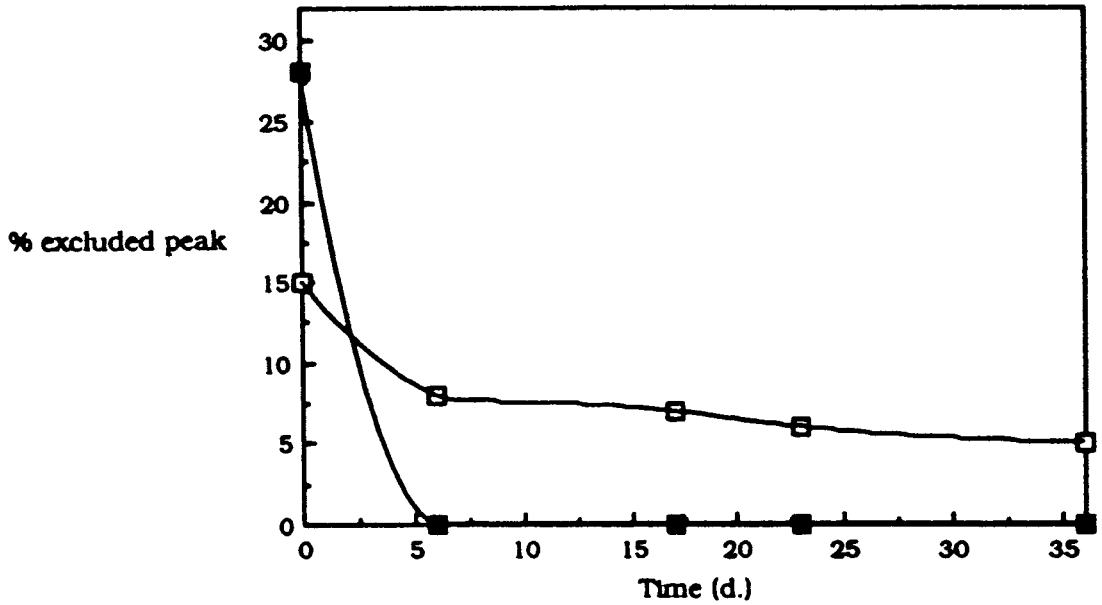


Figure 9 Stability of excluded peak of aggregates in water at 37°C with (■) percent excluded peak at 37°C and (□) percent excluded peak at 4°C. ODN sequence: 5′-XGTCCTTCTGGCTGTTC-3′.

than in 95% DMSO and the degradation was faster at 37°C than at 4°C (Fig. 9).

The above results suggest that the formation of aggregates was due to noncovalent bonds, because the process was slow (kinetics studies) and reversible in water. In DMSO the dielectric constant was probably not high enough to ensure the breakage of fairly weak bonds, which explains why the excluded peak did not disappear in this solvent.

Other Factors Involved in Aggregate Formation

The size of the synthetic macromolecule also played a significant role on this aggregation phenomenon. Indeed, the SEC analyses of the PMAMVE 1A sample never revealed the presence of excluded peaks as the PMAMVE 1 sample did. This led us to suppose that longer polymer chains would favor reticulation due to high flexibility and chain interpenetration.

No excluded peak was ever observed at any polymer and ODN concentrations with the PMAMVE 1A polymer sample of lower molecular weight. This was evidence that polymer chain interactions might be key factor in the formation of superstructures.

The last factor was the oligonucleotide base composition, but that will be dealt with in a future study.

CONCLUSIONS

In this work the experimental conditions were determined to afford a high rate of immobilization of ODNs to MA copolymers, and we analyzed the reaction products in an attempt to understand the phenomena implied in their formations.

The coupling reaction was found optimal in a mixture containing 5% 0.1*M* borate buffer and 1*M* NaCl in DMSO. The solvent effect on the course of the coupling reaction was drastic; DMF, for instance, gave no coupling reaction. The pH of the buffer should not exceed 9.3, otherwise the hydrolysis side reaction would be favored, hence reducing the coupling yield. Finally, the coupling reaction was a very fast process and reached completion in less than 10 min.

SEC-MALLS of the conjugates showed that highly reticulated aggregates (high molecular weights, low gyration radii) were formed during coupling of the ODN to polymer sample PMAMVE 1. A kinetics study demonstrated the crosslinking process to be much slower than the grafting step. This observation, associated with the lack of stability of these aggregates when stored in water, led to the assumption of a nonchemical reason for the occurrence of the crosslinking. Nevertheless, it is difficult to rule out the chemical origin of the crosslinking due to the fact that non-amino-modified ODN can also be grafted onto the polymer, proving that ODN borne amine moieties are capable of reacting with anhydride groups of the copolymers.

For this crosslinking to occur, polymer chain interactions were required; with a small polymer chain length sample, no crosslinking was detected by SEC.

Further work is in progress in the lab to address the issue of the effect of the base sequence of oligonucleotides on the course of the reaction and physicochemical properties of the polymer–ODN conjugates.

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