2366–2369 Nucleic Acids Research, 1994, Vol. 22, No. 12

Enhancement of selectivity in recognition of nucleic acids via chemical autoligation

Sergei M.Gryaznov*, Ronald Schultz[1], Surendra K.Chaturvedi[1] and Robert L.Letsinger[1]
Lynx Therapeutics Inc., 3832 Bay Center Place, Hayward, CA 94545 and [1]Department of Chemistry, Northwestern University, Evanston, IL 60208, USA

Received February 17, 1994; Revised and Accepted May 2, 1994

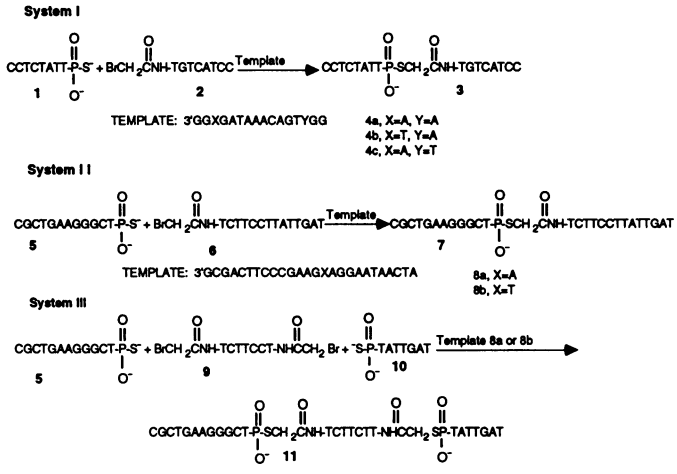
ABSTRACT

A new approach to increase the selectivity of interaction between oligonucleotide probes and target nucleic acids is described. In place of a single, relatively long oligonucleotide probe, two or three short oligomers terminated by thiophosphoryl and bromoacetamido groups are employed. Fast and efficient autoligation takes place when the oligomers hybridize in a contiguous mode to the same complementary strand such that a thiophosphoryl group on one strand and a bromoacetamido group on another are brought into proximity. A single nucleotide mismatch for the short probes leads to marked reduction in the rate of autoligation. The binding affinity of the product is close to that for a natural probe of the same length. This approach could have potential in oligonucleotide-based diagnostics, chemical amplification systems, and therapeutic applications.

INTRODUCTION

Synthetic oligonucleotides are being extensively used as sequence-specific antisense agents (1–3), as well as probes for hybridization based detection assays of nucleic acids (4, 5). One of the problems remaining to be resolved, and especially relevant to antisense applications, concerns the formation of complexes with targets that are not fully complementary. Relatively long oligomers (e.g. ~20-mers) are needed to provide a unique sequence and a high binding affinity in targeting human genetic segments; however, the longer oligomers are not highly selective as antisense agents (6). Short oligomers (e.g. 7–12 mers), which bind with low affinity, are much more effective in discriminating between complementary strands and targets containing one or more mismatches. We describe here an approach designed to capitalize on the positive features of both the longer and the shorter oligomeric agents (7). It is based on *in situ* chemical ligation of relatively short oligonucleotide fragments bearing electrophilic bromoacetamido and nucleophilic phosphorothioate monoester groups at their termini. As previously reported, rapid and efficient autoligation (ligation without condensing agents) takes place when such oligomers hybridize in a contiguous mode to a complementary strand (8).

In testing the effects of mismatches in target sequences on autoligation of relatively short oligonucleotides, we have examined the efficiency of coupling in the following three systems.



EXPERIMENTAL SECTION

General methods

Ion exchange high performance liquid chromatography (IE HPLC) was carried out on a Dionex Omni Pak NA 100 4×250 mm column at pH 12 (10 mM NaOH) with a 2%/min gradient of 1.5 M NaCl in 10 mM NaOH; 1 mL/min flow rate. For analyses in System I, the gradient solution also contained MeCN (2% by volume). Reversed phase (RP) HPLC was carried out with a Hewlett Packard Hypersil ODS 5 μm, 4.6×200 mm column at pH 7 in aq. 30 mM Et_3N/HOAc with a 1%/min gradient of MeCN. Polyacrylamide gel electrophoresis (PAGE) was carried out with denaturing cross-linked 20% polyacrylamide gels (5% bis-acrylamide); Rm values are relative to xylene cyanol. Except where otherwise noted, melting curves were obtained using solutions 0.10 M in NaCl, 5 μM of each oligonucleotide, pH 7.0, by following changes in absorbance at

*To whom correspondence should be addressed

260 nm while ramping the temperature at a rate of 1°C/min. Oligonucleotides were prepared via automated synthesis using standard cyanoethyl phosphoramidite chemistry. They were isolated with the DMT group intact, and, following detritylation (80% aq. HOAc, 15 min), were purified by successive RP HPLC and IE HPLC.

Preparation of CCTCTATT-P(O)(OH)S-

This oligonucleotide 3'-phosphorothioate was prepared by the procedure described in reference 9. It was also prepared conveniently starting with '3'-Phosphate CPG' supplied by Glen Research [DMTO$(CH_2)_2SO_2(CH_2)_2$O-succinyl-lcaa-CPG]. In the latter case the DMT group was removed by DCA treatment and the support was phosphitilated with a DMT−thymidine cyanoethylphosphoramidite reagent. Following sulfurization with a 5% solution of S_8 in CS_2/pyridine (1/1 v/v) (45 min reaction) the oligonucleotide was synthesized by standard protocol. After isolation by preparative HPLC, the product eluted as a clean peak on RP HPLC (16.7 min) and on IE HPLC, pH 12 (16.4 min).

Table 1. Properties of oligonucleotides

Oligomers	R min[a]	Rm[b]	Tm °C[c]	Tm °C[d]
5	22.4	1.54	54.3	54.3
6	21.7	1.32	40.8	31.8
7	26.4	0.83	65.3	59.0
9	16.6	1.70	18.3	<5
10	18.4	1.86	17.0	17.0
11	26.5	0.81	63.0	52.2[e]
12[f]	26.3	0.85	66.8	60.0

[a]Retention time on IE HPLC, pH12.
[b]PAGE, mobility relative to xylene cyanol.
[c]Tm for duplex with complementary template **8a** in 20 mM MES and 20 mM $MgCl_2$, 1 μM in each oligomer.
[d]Tm for duplex with mismatched template **8b** under same conditions as in footnote c.
[e]Very low hyperchromicity (~7%) observed for this curve.
[f]Oligomer **12** is a natural phosphodiester compound possessing the same nucleotide sequence as **7** and **11**.

It formed a complex with template **4a** exhibiting Tm 23°C (0.2 M NaCl); interaction with mismatched template, **4b**, under the same conditions was very weak (Tm <2°C). In support of the presence of a terminal thiophosphoryl group, oxidation of 1 A260 unit of the oligomer in 10 μL of water with 1 μL of 1 M aq. $K_3Fe(CN)_6$ (3 hr at 4°C) afforded a product eluting later on IE HPLC, as expected for oxidation of a terminal oligonucleotide phosphorothioate to a dimeric disulfide derivative (9).

Preparation of $BrCH_2C(O)NH$-TGTCATCC

To 5 A_{260} units of NH_2-TGTCATCC (prepared as in ref. 10; elution time 16.5 min for IE HPLC at pH 12; Tm = 32°C with template **4a**, Tm = 15°C with mismatched template **4b**) in 15 μL of 0.2 M sodium borate buffer, pH 8.5, at room temperature was added 15 μL of 0.4 M N-succinimidyl bromoacetate in MeCN. After 30 min the mixture was diluted to 0.5 mL with water and desalted on a NAP-5 column. The bromoacetamido derivative (3.5 A_{260} units) was isolated by RP HPLC followed by lyophilization. It was homogeneous (~99% as a single peak) as judged by RP HPLC and by IE HPLC at pH 7 (30 mM Et_3N/HOAc, 10% MeCN with a 2%/min gradient of 1.0 M NaCl, 30 mM Et_3N/HOAc, 10% MeCN). When analyzed by IE HPLC at pH 12, however, two peaks appeared—a major peak (~75%) at 15.4 min and a minor one (~25%) at 14.5 min. Rechromatography of the product collected from the major peak again yielded the two peaks in about the same ratio. We conclude that the minor peak is a degradation product (probably the hydroxyacetamido derivative) formed on exposing the bromoacetamido derivative to the strongly alkaline solution (pH 12) used in the chromatography. In agreement with this conclusion, the percentage of material in the faster eluting material increased to ~60% and then to >90% when the bromoacetyl derivative was exposed to a solution at pH 12 for 1 hr and for 2.7 hr, respectively, before chromatography.

Compounds for Systems II and III

These compounds were prepared as described for the oligomers in System I and in reference 8−10. Some physico-chemical

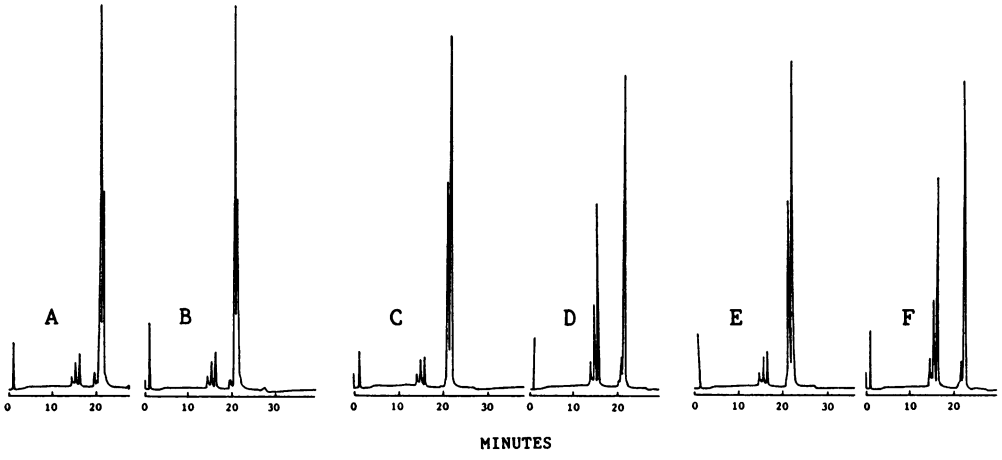


Figure 1. IE HPLC of products from System I (**1** + **2** + template **4a**, **4b**, or **4c**) in buffer I, 5 min reaction time: A, with **4a** at 0°C; B, with **4a** at 30°C; C, with **4b** at 0°C; D, with **4b** at 30°C; E, with **4c** at 0°C; F, with **4c** at 30°C. The peaks and assignments for A and B are, respectively: ~15.3 and 14.5 min (bromoacetamido derivative 2 and its hydrolysis product formed during analysis); 16.5 min (phosphorothioate **1**); 20.9 min (template **4a**); 21.5 min (product **3**). The assignments are the same for figures B−F except templates **4a** and **4b** elute at ~22.5 min, after the product peak. The recorder sensitivity was 0.5 for A, B and 0.1 for C−F.

characteristics of the oligonucleotides are summarized in Table 1. Oligomer **6** proved to be considerably more stable than oligomer **2** in solution at pH 12. On direct IE HPLC at pH 12 only about 3% was hydrolyzed, and after standing in a pH 12 solution for 4 hr, chromatography at pH 12 showed only about 12% conversion to the faster eluting product. The hydrolysis rate of the bromoacetyl derivative is therefore related to the oligonucleotide sequence, and in the case of oligomer **2** may be enhanced by the proximity of a guanine residue.

Ligation experiments

Ligations were carried out with 1 μM solutions of each oligomer in an aqueous solution (buffer I: 15 mM sodium phosphate and 0.2 M NaCl, pH 7.0; or buffer II: 20 mM MES and 20 mM $MgCl_2$, pH 7.05; or buffer III: 0.1 M NaCl). At the end of the designated time an aliquot was injected in the HPLC unit for IE chromatography at pH 12. This pH was selected to denature oligonucleotide complexes in the system. The structure assigned to ligation product **3** is supported by the elution time on IE HPLC (close to values for 16-mers **4a–c**) and the melting curve for the complex of **3** with **4a**; Tm = 56°C for 4 μM of the complex in 0.1 M NaCl. Under the same conditions Tm = 62°C for a natural phosphodiester duplex with the same nucleotide sequence. In addition to the elution time on IE HPLC, mobility on gel electrophoresis, and Tm value for thermal dissociation of the complex formed with a complementary oligonucleotide (see Table 1), the structures assigned to compounds **7** and **11** were supported by selective cleavage of the -$NHCOCH_2SPO_2$- links with NaI_3 by the procedure described in reference 8.

RESULTS AND DISCUSSION

Ligation studies with CCTCTATT-OP(O)(OH)S^- (**1**) and $BrCH_2C(O)$NH-TGTCATCC (**2**) (System I) were carried out in solutions 1 μM in each oligomer at pH 7.0 (15 mM sodium phosphate and 0.2 M NaCl). Products were analyzed by ion

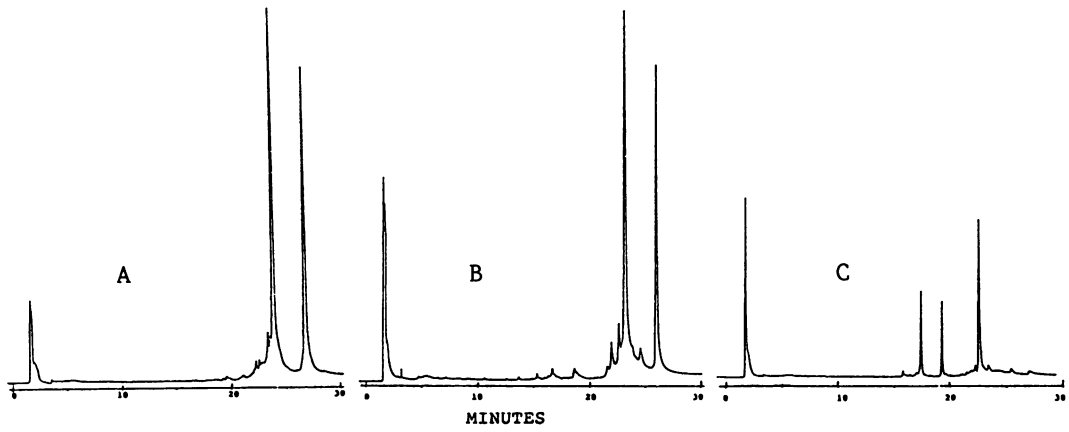


Figure 2. IE HPLC of products formed in 5 min in buffer II at 0°C for: A, Systems II (**5** + **6** + template **8a**); B, System III (**5** + **9** + **10** + template **8a**); C, System III without any template (**5** + **9** + **10**). The peaks at ~23 and 25 min correspond to template **3a** and ligation product **7** or **11**, respectively; the peaks at ~17, 18, and 22 min correspond to oligomers **10**, **9**, and **5**.

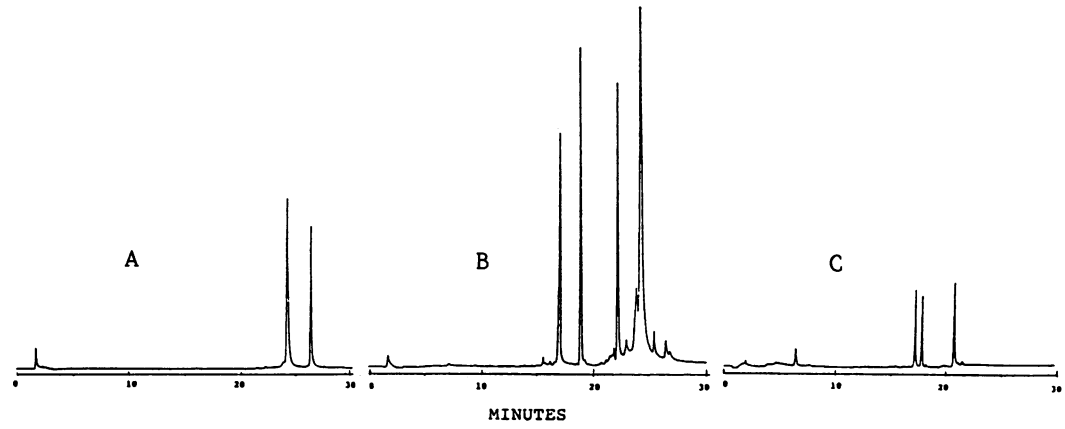


Figure 3. IE HPLC of products formed after 2 hr in buffer III at 23°C for: **A**, System III with fully complementary template **8a**; **B**, System III with mismatched template **8b**. **C**, Products of cleavage of **11** with NaI_3 followed by DTT according to reference 9; note that **11**, which elutes at ~26.5 min, was transformed to three oligonucleotide cleavage products, eluting at ~17, 18, and 20 min.

exchange HPLC at pH 12. In absence of a template, no ligation was observed for reactions carried out for 45 minutes at either 0°C or 30°C. In marked contrast, rapid ligation occurred when an appropriate template was present. HPLC profiles are shown in Figure 1 for reactions carried out for 5 minutes at 0°C or 30°C in presence of a template (~10 % excess template) that was either fully complementary or contained one mismatch. The tallest peak in each profile corresponds to the template. The ligation product is the peak eluting immediately after the template (compound **4a**) in Figure 1A,B and just before the template (compound **4b** or **4c**) in Figure 1C,E. Residual thioate (**1**) appears at 16.5 minutes and the residual bromoacetamido derivative appears as a double peak at 14.5 and 15.3 minutes (the 14.5 peak is formed during chromatography at pH 12; see experimental section). At 30°C the ligation efficiency is markedly dependent on proper base pairing. From the peak areas we estimate that the conversion of **1** and **2** to **3** was approximately 75% when the fully matched template (**4a**) was present (Figure 1B), but ligation amounted to less than 5% when a single mismatch occurred in a segment of the template binding to either oligomer **1** (Figure 1D) or to oligomer **2** (Figure 1F). Proper base pairing proved to be much less important at 0°C, where the binding affinity is much greater even when the template has a mismatched base. At this temperature, conversion to the ligated product amounted to 85%, 78%, and 74% for mixtures containing **4a** (matched template, Figure 1A) and **4b**, and **4c** (mismatched templates, Figure 1C,E), respectively (11).

These results are supported and extended by work with another sequence involving double (System III) as well as single ligation (System II) of the modified oligonucleotides. After 5 minutes of ligation in buffer II (20 mM MES containing 20 mM $MgCl_2$) at 0°C, yields of the 26-mer products of ligation from System II (**7**) and System III (**11**) were ~97% (Figure 2A,B). Practically no ligation was observed in System III when the template was absent (Figure 2C). Ligation on mismatched template **8b** at 0°C produced **7** and **11** in yields of 97% and 67% respectively, probably as a consequence of formation of partially complementary complexes. Better discrimination was achieved in low salt buffer III (0.1 M NaCl) and at a higher annealing temperature. Only 18% of ligation product **7** was observed in System II after 2 hours of reaction with mismatched template **8b** at 37°C; at 41°C the yield was only 7%. Under the same conditions but with fully complementary template **8a** the yield was ~98% at both temperatures.

In the four component system (III) at 23°C, only ~5% of product **11** was detected after 2 hours incubation with mismatched target **8b**, in contrast to 94% for the fully complementary template, **8a** (Figure 3A,B). These data indicate that formation of a duplex is important for an efficient ligation reaction. In Systems II and III a very small fraction of oligomers are in the duplex state with mismatched template **8b** at 41°C and 23°C (Tm's 31.8°C and <5°C; Table 1); therefore low yields of ligation products are obtained. In contrast, a substantial fraction of molecules hybridize with fully complementary template **8a** under analogous conditions (Tm's 40.8°C and 18.3°C; Table 1), resulting in formation of ligation products **7** and **11**.

In summary, this approach provides an effective means for discrimination of duplexes containing even a single mismatched base pair and could potentially increase the specificity of oligonucleotide ligation amplification techniques (12) or antisense applications.

ACKNOWLEDGEMENTS

The work at Northwestern University was supported by research grants from the National Cancer Institute and the National Institute of Allergy and Infectious Diseases (Grant UOI AI24846) and the National Institute of General Medical Sciences (Grant 10265).

REFERENCES

1. Helene, C., & Toulme, J. (1990) Biochem. Biophys. Acta. 1049, 99–125.
2. Uhlman, E., & Peyman, A. (1990) Chem. Rev. 90, 544–548.
3. Crooke, S.T., & Lebleu, B., Ed. (1993) Antisense Research and Application. CRC Press, Boca Raton, Fl.
4. Lowe, J.B. (1986) Clinica Chimica Acta, 157, 1–32.
5. Urdea, M.S., Warner, B.D., Running, J.A., Stempien, M., Clyne, J., & Horn, T. (1988) Nucleic Acids Res. 16, 4937–4956.
6. Wolf, T.M., Melton, D.A., & Jennings, C.G.B. (1992) Proc. Natl. Acad. Sci. USA, 89, 7305–7309.
7. A related approach employing different coupling chemistry was described by Goodwin, J.T., & Lynn, D.G. (1992) J. Am. Chem. Soc. 114, 9197–9198.
8. Gryaznov, S.M., & Letsinger, R.L. (1993) J. Am. Chem. Soc. 115, 3808–3809.
9. Gryaznov, S.M., & Letsinger, R.L. (1993) Nucleic Acids Res. 21, 1403–1408.
10. Gryaznov, S.M., & Letsinger, R.L. (1993) Tetrahedron Lett. 34, 1261–1264.
11. When the reaction was carried out with a slight excess of the bromoacetamido derivative, **2**, the yield of ligated product based on consumption of the limiting reagent, **1**, was 94% and 97% for 4 min ligation on matching template **4a** at 0°C and at 22°C, respectively.
12. Weiss, R. (1991) Science, 254, 1292–1293.