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Selection of Modified Oligonucleotides with Increased Target Affinity via MALDI-Monitored Nuclease Survival Assays

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Reported here is how modified oligonucleotides with increased affinity for DNA or RNA target strands can be selected from small combinatorial libraries via spectrometrically monitored selection experiments (SMOSE). The extent to which target strands retard the degradation of 5′-acyl-, 5′-aminoacyl-, and 5′-dipeptidyl-oligodeoxyribonucleotides by phosphodiesterase I (EC 3.1.4.1) was measured via quantitative MALDI-TOF mass spectrometry. Oligonucleotide hybrids were prepared on solid support, and nuclease selections were performed with up to 10 modified oligonucleotides in one solution. The mass spectrometrically monitored experiments required between 120 and 300 pmol of each modified oligonucleotide, depending on whether HPLC-purified or crude compounds were employed. Data acquisition and analysis were optimized to proceed in semiautomated fashion, and functions correcting for incomplete degradation during the monitoring time were developed. Integration of the degradation kinetics provided "protection factors" that correlate well with melting points obtained with traditional UV melting curves employing single, pure compounds. Among the components of the five libraries tested, three were found to contain 5′-substituents that strongly stabilize Watson−Crick duplexes. Selecting and optimizing modified oligonucleotides via monitored nuclease assays may offer a more efficient way to search for new antisense agents, hybridization probes, and biochemical tools.

Introduction

Modified oligonucleotides are used as antisense and antigene inhibitors of gene expression,[1] hybridization probes, PCR primers, and biochemical tools.[2] For the development of modified oligonucleotides, unmodified, natural oligomers of DNA or RNA usually provide the lead compounds. Improving the properties of these lead compounds toward a desired application is commonly achieved via rational design of modifications and iterative optimization. For some properties, such as luminescence or reactivity toward a given functional group of the target, the design of a modification is straightforward and usually requires no more than an appendage without target-specific binding properties. For other properties, such as increased affinity for a nucleic acid target, the choice of a chemical modification is less obvious, particularly when both target selectivity and nuclease resistance are desired. High-affinity analogues, such as PNAs,[3−5] are structurally quite different from DNA and thus difficult to design from the lead. Others, such as locked nucleic acids, were designed to bind with reduced entropic cost, but are not always found to do so.[6] Some dimethylenesulfone-linked analogues of RNA, on the other hand, do not bind to complementary RNA strands at all,[7] even though they are known to form Watson−Crick duplexes,[8] most probably due to difficult-to-predict competing structures.[9] Where the design becomes difficult, lead optimization may benefit from methodologies allowing for a rapid search of structure space for derivatives with improved properties. This is particularly true for binders to structured RNA or DNA targets, where readily designable Watson−Crick and Hoogsteen base pairing interactions are of limited usefulness.[10] Combinatorial synthesis and high-throughput screening are methodologies for rapidly searching structure space.

Even though RNA was one of the first classes of compounds for which combinatorial syntheses and in vitro selections were established,[11] combinatorial syntheses of *modified* oligonucleotides are not common. This may be due to the fact that phosphoramidites of modified nucleosides are not easy-to-prepare building blocks. For non-phosphodiester-linked analogues, a lack of routine solid-phase assembly protocols for oligonucleotides of any sequence complicates the exploration of structure space via combinatorial synthesis. Further, there are no well-established high-throughput screening techniques for modified short oligonucleotides other than PNAs.[12,13]

Out of an interest in oligonucleotides with increased target affinity and selectivity, we and others have recently reported synthetic methodologies for the synthesis of oligonucleotide-peptide hybrids,[14] particularly linker-free hybrids.[15−17] These allow an easier entry into combinatorial techniques than entirely phosphodiester-based compounds, as they access the large structure space of commercially available amino acid-, acyl-, and PNA-building blocks, and can be assembled

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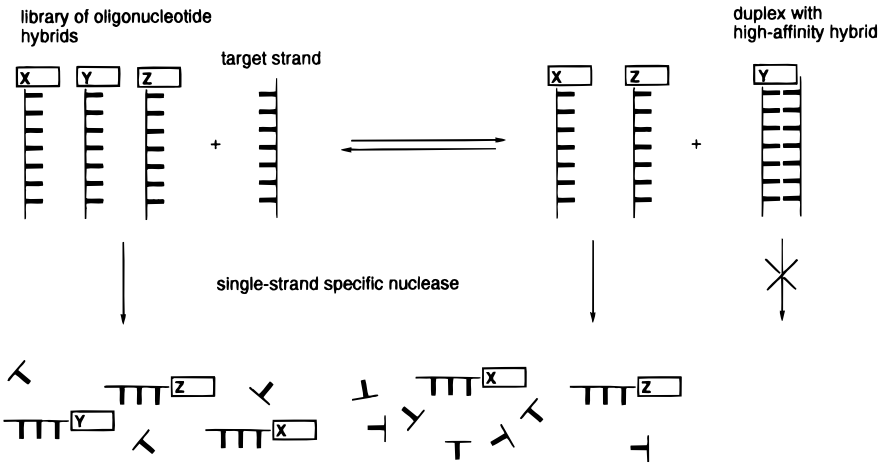


Figure 1. Illustration of nuclease survival selection experiment. Selection of oligonucleotides whose non-nucleic acid terminus increases the affinity for the target strand is based on a higher propensity to be in a duplex state and, thus, unavailable for nuclease attack. Kinetics of nuclease degradation are measured via quantitative MALDI-TOF mass spectrometry.

entirely on solid supports. Both 5′- and 3′,5′-modified oligonucleotides can be prepared as small chemical libraries or chemsets,[15,16,18] and increased target affinity[19] and selectivity[20,21] have been demonstrated for hybrids, as well as increased exonuclease stability.[16]

We now present a new technique that allows the selection of high-affinity binders from libraries of modified oligonucleotides. This technique relies on spectrometrically monitored selection experiments (SMOSE).[22−24] The spectrometric technique employed is MALDI-TOF mass spectrometry[25] of oligoribonucleotides,[26] under conditions optimized for quantitative detection.[27,28] Selection among the modified oligonucleotides is achieved by subjecting them to a single strand-specific nuclease under conditions where the higher affinity compounds can form duplexes to a greater extent than low-affinity competitors. We have used this technique to select compounds whose 5′-terminus stabilizes duplexes with strands complementary to the DNA portion by interacting with the exposed nucleobases of the terminal base pair.

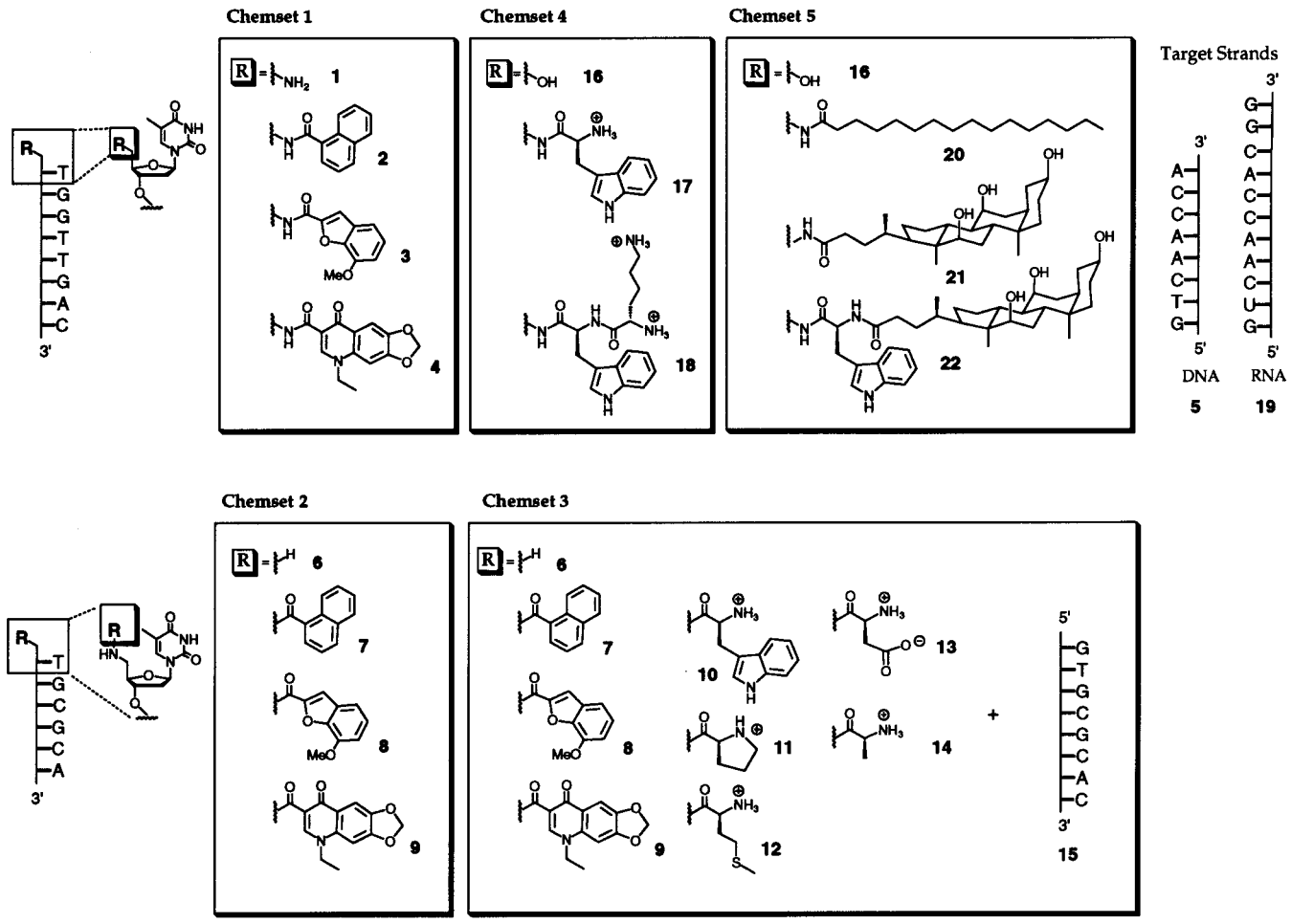
Results

Our search for 5′-terminally modified oligonucleotides with increased target affinity started with the synthesis and evaluation of single compounds. We use automated solid-phase DNA synthesis for assembly of the DNA portion of our molecular hybrids and Fmoc-peptide synthesis protocols for the non-nucleic acid portion.[15] The entire synthesis is performed on solid support. Mixed sequence oligonucleotides containing all four nucleobases, both self-complementary and non-self-complementary, were 5′-modified with a wide range of modifications to test our methodology. Lipophilic aromatic and aliphatic moieties were included in the pool of building blocks, together with all proteinogenic amino acids except cysteine and histidine. The first compounds to be tested were HPLC purified individually and the duplexes with their target strands subjected to UV melting experiments.[15,19] This allowed the testing of up to one compound per day.

Simultaneously monitoring the hybridization of three oligonucleotides to a solid-phase bound target strand by quantitative MALDI-TOF mass spectrometry increased the efficiency of the testing, but was still very time-consuming and limited to solutions with high salt conditions.[28] We therefore designed the assay schematically shown in Figure 1. This assay also relies on quantitative MALDI-TOF mass spectrometry[27] as the monitoring technique, but the binding between oligonucleotides and their target occurs in solution, where the hybridization kinetics are faster and no surface effects can distort the binding equilibrium. The selection of high-affinity binders with this assay relies on their prolonged survival when incubated with a single-strand specific nuclease. A mixture of oligonucleotides with the same DNA portion, but differently modified termini, is allowed to compete for a target strand. In the resulting binding equilibrium, the oligonucleotides with increased affinity for the target will be found in the double-stranded state to a greater extent than their competitors, and thus they will be less accessible to nuclease attack. When an exonuclease attacking at the 3′-terminus is used, the attack occurs at the unmodified terminus, where all competitors offer the same structure, avoiding any bias not based on target affinity. The degradation kinetics of the competing oligonucleotides are measured simultaneously by MALDI-TOF mass spectrometry, and a structure−activity map for a small library is obtained in one experiment. Since the differently modified oligonucleotides can be detected selectively, the nuclease selection can be performed with crude products of the solid-phase synthesis, without removal of possible failure sequences. Our synthetic method usually produces crude products sufficiently pure to show the product as the most prominent peak in the MALDI mass spectrum.[15,16]

To test the indirect affinity selection, we first performed a series of assays where a single 5′-acylated derivative of the 5′-amino-5′-deoxy-DNA sequence 5′-TGGTTGAC-3′ competed with its unacylated precursor for the target DNA strand (data not shown). These experiments were performed with snake venom phosphodiesterase (SVP, phosphodiesterase I, EC 3.1.4.1) and provided us with a set of four oligonucleotides (**1**−**4**, Scheme 1) whose degradation was either slightly faster (**2**) or slower (**3** and **4**) than that of control oligonucleotide **1**. These compounds were then purified and individually tested in UV melting curves with

Scheme 1



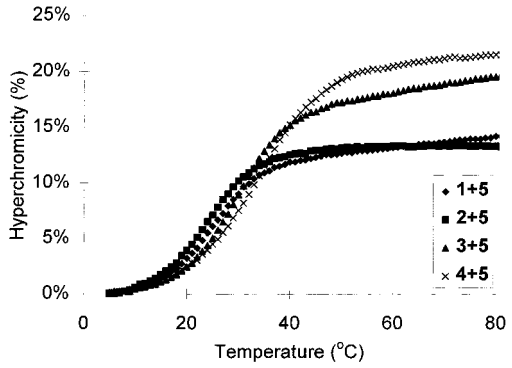


Figure 2. UV melting curves of modified oligonucleotides **1**−**4** with target strand **5**, acquired at 1.1 μM strand concentration under the same buffer conditions as those used for the nuclease selection (75 mM NH_4OAc buffer, pH 6).

target strand **5** (Figure 2). The order of melting points (**4** > **3** > **1** > **2**) agreed with the relative nuclease resistance in the presence of the target strand, encouraging us to pool compounds **1**−**4** into **chemset 1** and to extend our assay to multicomponent selections.

First, it was tested whether the entire **chemset 1** and target strand **5** could be simultaneously detected via quantitative MALDI-TOF mass spectrometry with our internal standard-based method.[27,28] Solutions of crude **2**−**4** were quantified via UV spectroscopy, using extinction coefficients calculated for the pure compounds. These were pooled to give 11.75 μL of a solution containing 300 pmol of each crude oligonucleotide and 1 equiv of HPLC-purified target strand **5**. Control oligonucleotide **1** was present in the solution as a low-level side product from incomplete coupling reactions. If the combined quantities of **1** in the crude of all three hybrids **2**−**4** together were not sufficient to give a detectable MALDI peak for **1**, coupling times were shortened or additional HPLC-purified **1** was added to get a signal intensity comparable to that of the hybrids. A series of dilutions were prepared in 75 mM ammonium acetate buffer, and MALDI spectra of 1 μL aliquots were acquired with matrix preparations containing 19.3 pmol of DNA decamer 5′-CGCATTAGCA-3′ as internal standard. The decamer was chosen since its molecular weight is sufficiently high to allow detection of its $n-1$ degradation fragment in the spectrometric window between the full length oligomer and the highest molecular weight hybrid. Thus any (undesired) nuclease degradation occurring after removal of the aliquot from the nuclease assay can be detected. Calibration plots are shown in Figure 3. Data points are the averages of the relative signal intensities (analyte/internal standard) of four spectra with their standard deviations, and lines are linear fits. While not completely without outliers, the calibration plots obtained are satisfactory for quantitative monitoring during nuclease degradation. It is important to note that such results were only obtained with the previously reported conditions for acquisition of spectra,[22,27,28] i.e., with a laser

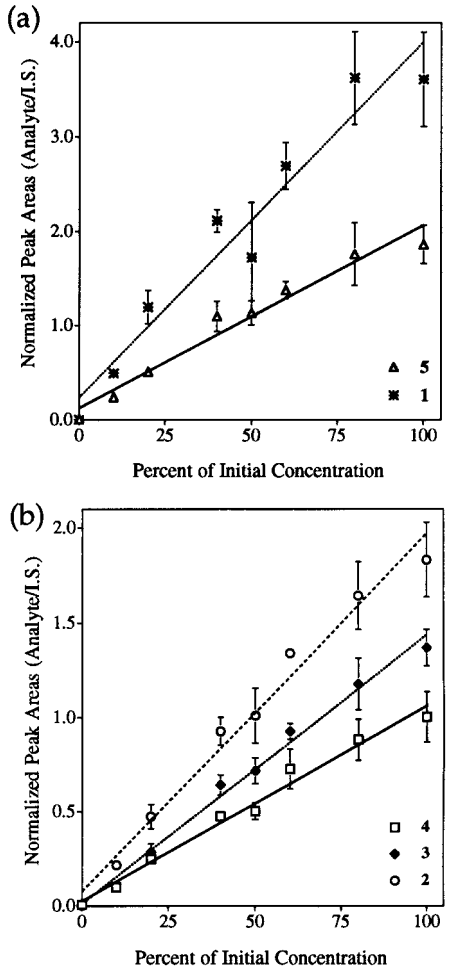


Figure 3. Calibration plots for detection of oligonucleotides **1**−**5** via MALDI-TOF mass spectrometry, measured with 300 μmol of the crude oligonucleotides as the initial concentration as determined by UV spectrophotometry using the extinction coefficient for the pure full length compound. Data points are averages of peak integral ratios of four spectra + standard deviation. Lines are least square fits to data points. Oligonucleotides without (a) and with (b) 5′-substituents are plotted separately for clarity.

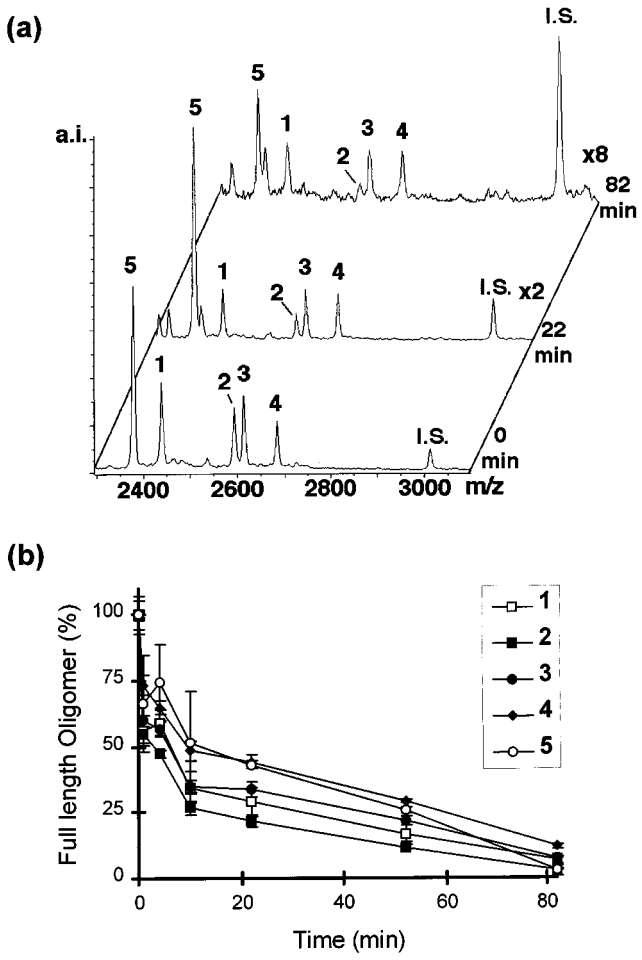


Figure 4. Nuclease selection from **chemset 1** using snake venom phosphodiesterase and target strand **5**. (a) Representative MALDI-TOF mass spectra from the monitored nuclease selection assay. The unlabeled peaks in spectra at time points 22 and 82 min are heptamer oligonucleotide degradation products from **2**−**4**. (b) Kinetics of the degradation reactions. Data points are averages of relative signal intensities (analyte/internal standard, I.S.) from four spectra normalized to the signal obtained at $t = 0$ min as 100%. Error bars are ± one standard deviation; a.i. = arbitrary intensity.

power well above the desorption limit, exhaustive or deep ablation, and discarding of spectra without a minimum number of ion counts for the internal standard.

Representative MALDI spectra from the nuclease survival selection with **chemset 1** using phosphodiesterase I, are shown in Figure 4a. This experiment was performed with 11.75 μL of the same solution containing 300 pmol of the crude of each oligonucleotide hybrid that was used for the calibration experiment described above, except that 3.2×10^{-4} units of nuclease were added. As desired, no degradation of the internal standard was observed, indicating that the mixing of the aliquots from the assay solution with the chilled matrix fully quenched the nuclease reaction, avoiding unspecific degradation in the ethanol-containing (and thus denaturing) matrix mixture. 5′-Modified acyl-oligonucleotide hybrid **4**, with the least prominent peak at the beginning of the assay, withstood the nuclease attack to the greatest extent, as more clearly discernible after plotting the degradation kinetics (Figure 4b). As expected from the exploratory single-component assays and the melting curves, the highest affinity hybrid **4** was degraded more slowly than **3**, which in turn withstood attack longer than control oligomer **1** and naphthoic acid residue-bearing competitor **2**. The kinetics appear to be biphasic, with a rapid first phase and a slower second phase, probably rate-limited by the off-rate for any given modified oligonucleotide engaged in duplex formation with **5**. In the absence of altering on-rates, changes in the off-rates, of course, alter the equilibrium constant.

In principle, the ratio of duplexes formed in the initial binding equilibrium may be obtained from the rate constants for the first, fast phase of the degradation. Rate constants obtained from biexponential fits to the seven data points obtained in a typical assay are, however, associated with large experimental errors. Fitting to the late data points can be complicated by the appearance of the shorter fragments of the modified oligonucleotides. These not only have different K_M and k_{cat} values but may become inhibitors as the nuclease approaches the modified 5′-terminus. To quantify the retardation of the degradation for each oligonucleotide, we therefore decided to integrate the area under the point-to-point fit of the data points in the normalized kinetics. The ratio of the area under the degradation curve of a given test

Table 1. Protection Factors Obtained from Monitored Nuclease Selection from **Chemset 1** at 23 °C

compd	target	equivalents complement	PF[a]	$PF_{co+trunc}$[b]
1	**5**	1	1	1
2	**5**	1	0.74	0.61
3	**5**	1	1.13	1.23
4	**5**	1	1.48	1.89
5	**1**−**4**	4	1.38	1.74

[a] Protection factor. [b] Protection factor after correction with a cutoff of 12% residual full length oligomer and truncation correction. See text and Supporting Information for definition of protection factor and correction functions.

compound versus that of the control oligonucleotide **1** was defined as a "protection factor" (PF). Protection factors for the nuclease selection from **chemset 1** are given in Table 1.

Since the protection factors are not known prior to the assay, it is impossible to choose the ideal reaction time at which the most resistant of the hybrids is just fully degraded at the end of the assay. Too long a reaction time produces only a few useful and many useless data points. Too short a reaction time biases the protection factors, as some degradation reactions will be complete, but others will be incomplete, leading to too low an integral in the protection factor calculation for the latter. Rather than optimizing the reaction time iteratively, i.e., running more than one assay for every selection, we developed a correction for incomplete degradation referred to as "cutoff and truncation" correction. This form of correction is explained graphically in Figure S1 of the Supporting Information. A "cutoff and truncation" correction produces protection factors from area integration down to an artificially elevated baseline where all components in the assay have seemingly reached full degradation, allowing for a fair comparison of their degradation kinetics. While often giving a better numerical correlation with UV melting points (vide infra), in no case did the correction change the nuclease stability ranking of compounds.

The entire analysis process was automated. Spectra were analyzed with an AURA script producing report files with peak integrals. These report files were then analyzed with a PERL script, converting them into normalized kinetics data and calculating PFs with and without cutoff and truncation correction. The source code of these scripts may be downloaded from the Internet site of the authors.

The degradation kinetics of target strand **5** in the selection from **chemset 1** (Figure 4) initially gave some reason for concern. If formation of nuclease-inaccessible double-stranded species was the basis of prolonged survival of **1**, **2**, and **3**, then **5** should survive to the same extent as the sum of the individual components that it may form a duplex with. This was not found to be the case. While **5** survived longer than **1**−**3**, (protection factor of 1.38), it was degraded more rapidly than **4**. Several control experiments were performed to find the cause of this phenomenon. The results of these experiments are summarized in Table 2.

First, a nuclease degradation without complementary strand **5** was performed. As expected for protection due to duplex formation, the protection factors were significantly decreased for **2**−**4**. The observation that the protection factors for **2** and **3** fell below unity demonstrates that their 3′-terminal nucleotides are slightly better substrates for the nuclease than that of **1**, helping to explain the low protection factors in the assay with **5**. Next, two experiments were performed with other than **1** equiv of **5**. The first, with 0.5 equiv, led to an increase in all protection factors (Table 2), as expected for a fiercer competition for the target strand, and a marked increase in the protection factor for **5**, as expected for a selection based on duplex formation and not single-strand protection. The second experiment, with 3 equiv of **5**, again demonstrated that duplex formation was causing protection from nuclease degradation, as the protection factors of **2**−**4** were all brought close to 1 under these less competitive conditions and that for **5**, now less well endowed with binders, dropped below unity.

Table 2. Protection Factors Obtained from Monitored Nuclease Selection from **Chemset 1** at 23 °C with Different Amounts of Target Strand **5**

compd	target	equivalents complement	PF[a]	$PF_{co+trunc}$[b]
		No Target		
1				1 (4)
2			0.56	0.52 (4)
3			0.71	0.66 (4)
4			1.14	1.17 (4)
		Substoichiometric Target		
1	**5**	0.5	1	nd
2	**5**	0.5	0.64	nd
3	**5**	0.5	1.04	nd
4	**5**	0.5	1.45	nd
5	**1**−**4**	8	2.47	nd
		Overstoichiometric Target		
1	**5**	3	1	1 (28)
2	**5**	3	0.85	0.70 (28)
3	**5**	3	1.20	1.19 (28)
4	**5**	3	1.28	1.38 (28)
5	**1**−**4**	0.75	0.53	0.23 (28)

[a] Protection factor. [b] Protection factor after cutoff and truncation correction. The cutoff in percent residual full length oligomer is given in parentheses; nd = not determined.

Analysis of the kinetics of formation and degradation of the first fragments of oligonucleotides in the original assay with 1 equiv of **5** (Figure 5) sheds light on why the target strand was not being protected to the extent of the sum of all binders: the heptamer formed from **5** reached its maximum concentration later than that of any of its competitors. This suggests that the heptamer cleavage product of **5** significantly contributes to the protection of **1**−**4** by forming exonuclease-resistant complexes with them. The relatively more rapid degradation of **5** than of the heptamer formed from it may either be due to the change from a purine to a pyrimidine as the 3′-terminal residue or due to the formation of a particularly stable complex with the shorter oligonucleotide target. Since we have previously observed similar degradation rates for oligonucleotide hybrids of different length, but with the same 3′-nucleobase,[22] the former may be more likely.

We next explored nuclease selections of acyl-oligonucleotide hybrids with increased affinity for DNA target strands from a chemset of 5′-modified self-complementary hexamers (**chemset 2**, Scheme 1). These have the advantage of

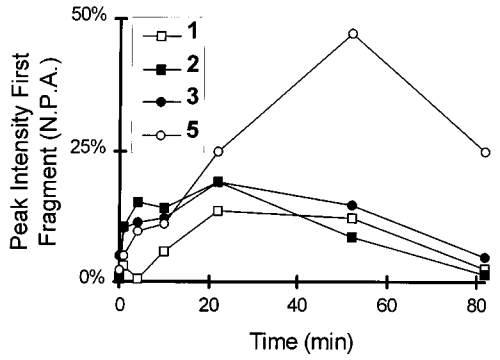


Figure 5. Kinetics of formation and degradation of the first degradation products of the components of **chemset 1** subjected to nuclease degradation, as shown in Figure 4. Relative peak intensities (analyte/internal standard) are plotted as normalized to the peak intensity (N.P.A.) of the respective full length oligomer at time t_0 of the assay. Error bars omitted for clarity. Compound **4** could not be quantified due to peak overlap.

allowing selections without a complementary strand, thus improving the signal-to-noise ratio for the remaining components in the spectra and decreasing the effort for the assays. Further, since symmetrical duplexes with twice the duplex-stabilizing effect of the 5′-appendage can form in the equilibrium, a greater selectivity in the assay may be expected, if the doubly stabilizing effect in the symmetrical duplexes is not offset by the "diluting" effect of mixed complexes with strands bearing poorly duplex-stabilizing 5′-moieties. Additionally, any stabilizing or destabilizing effect of the 5′-modifications can be expected to have a greater effect on the hexamer duplexes than on the octamer duplexes, where the portion of unmodified DNA is greater.

The results of the selection from **chemset 2** with snake venom phosphodiesterase are shown in Figure 6 and Table 3. The selectivity in the assay was indeed greater with the self-complementary oligonucleotides than that observed with **chemset 1** containing the same 5′-moieties. Even on the level of individual spectra, hybrid **9** could be identified as the sole survivor in the assay (Figure 6a). The order in the nuclease resistance of the hybrids was analogous to that found with non-self-complementary **chemset 1** (Figure 6b), but the difference between the protection factors was much greater, making the results easier to read and more significant over the experimental errors (Table 3). Control selection experiments with **chemset 2** at 10, 30, and 40 °C indicated that the initially chosen 23 °C, a value close to the melting point for **6**, gave the best selectivity in the assay (Table 3). This was not unexpected. Well below the melting point of the control, all hybrids in this self-complementary library are in the duplex form and nuclease protected. At much higher temperature, all duplexes are dissociated, again preventing selection for duplex stability. Only at an intermediate temperature, slightly below the melting point of the control duplex, are the high-affinity compounds mostly in duplex form (and thus nuclease protected) and the low-affinity compounds in single-stranded form (and thus nuclease accessible), allowing successful selection.

The next phase of the current work involved testing the nuclease selection assay thus developed with (i) a greater chemset size, (ii) a mixed DNA/RNA system, and (iii) a class

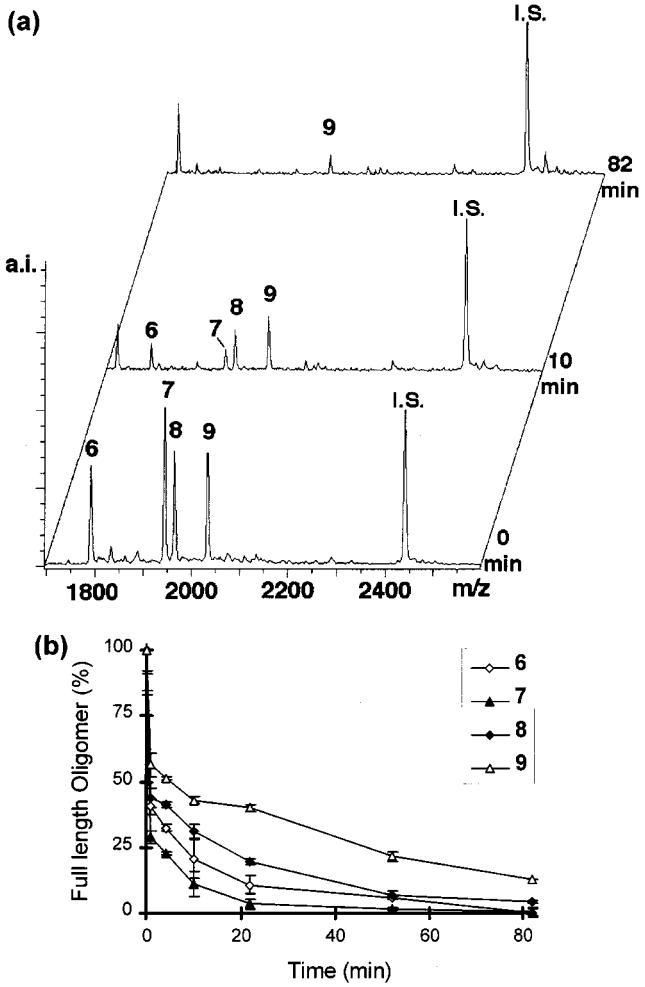


Figure 6. Nuclease selection from **chemset 2** with snake venom phosphodiesterase. (a) Representative MALDI-TOF mass spectra; unlabeled peaks in spectra from aliquots taken at 10 and 82 min reaction time are pentamer degradation products. (b) Kinetics of the nuclease reactions. Data points are averages of relative signal intensities (analyte/internal standard) from four spectra normalized to the signal obtained at t_0 as 100%. Error bars are ± one standard deviation; a.i. = arbitrary intensity.

of hybrids bearing large and strongly hydrophobic 5′-moieties (**chemsets 3**−**5**, Scheme 1). **Chemset 3** contains ten components: those four already contained in **chemset 2**, a chemset of the five aminoacyl-oligonucleotides **10**−**14** produced in a single mixed coupling reaction, and self-complementary octamer **15**. The latter was added to test for the formation of mixed duplexes, as the 3′-terminus of the longer oligonucleotide would present a dangling 3′-residue to the nuclease in a mixed duplex but would not do so in a symmetrical duplex with itself, where all nucleotides would be engaged in Watson−Crick base pairing. The nuclease selection experiment (Figure 7) showed that the formation of mixed complexes does indeed occur, as expected based on entropic considerations, since **15** was degraded more rapidly than any of the 5′-acylated hexamers, even though the melting point of its symmetrical duplex is 12.8 °C higher than that of **6**. Further, the successful selection of the quinolone-bearing hybrid **9** from this complex mixture with protection factors only slightly lower than those found with **chemset 2** under identical conditions (Table 4) demonstrated the efficiency of the selection assay. This is particularly so,

Table 3. Protection Factors Obtained from Monitored Nuclease Selections from **Chemset 2** at Different Temperatures

compd	T assay (°C)	PF[a]	$PF_{co+trunc}$[b]
6	23	1	1 (13)
7	23	0.52	0.48 (13)
8	23	1.50	1.99 (13)
9	23	2.86	5.66 (13)
6	10	1	1 (53)
7	10	0.80[c]	0.65 (53)
8	10	1.04[c]	1.12 (53)
9	10	1.15[c]	3.26 (53)
6	30	1	1
7	30	0.91	0.91[d]
8	30	1.57	1.57[d]
9	30	2.59	2.59[d]
6	40	1	1
7	40	0.85	0.85[d]
8	40	1.23	1.23[d]
9	40	2.00	2.00[d]

[a] Protection factor. [b] Protection factor after cutoff and truncation correction. The cutoff in percent residual full length oligomer is given in parentheses. [c] Small extent of degradation. [d] Full degradation, no cutoff and/or truncation required.

as all components, except **15**, were used as crude products from the solid-phase syntheses and the combined load of fragments from incomplete reactions decreases the signal-to-noise ratio in the spectra.

A selection with an RNA target strand was performed with undecamer **19** and **chemset 4**, a mixture of three compounds whose affinities for **19** had previously been determined via UV melting analysis.[19] The undecamer was chosen to mimic a longer biological target strand, for which both the best position for binding and the exact structure of the binder may have to be found.[29] When equilibrated with RNA **19** and subjected to degradation by SVP, the known highest affinity binder **18** was found to be the survivor in the MALDI spectra (Figure 8a). Nuclease survival of tryptophanyl derivative **17**, a known duplex-capping residue,[30] was prolonged over the control octamer **16** (Figure 8b), as expected based on the melting curve results.[19,30] The rapid degradation of the 3′-overhang of **19** again confirmed a selection based on sequestering of duplexes from nuclease attack, but it could also have been used to identify the binding site in a target whose preferred binding site was not known. Protection factors calculated from the kinetic data confirmed the clear order in nuclease survival noted in the spectra (Table 5).

Selection from **chemset 5** (Scheme 1) containing oligonucleotides **20**−**22** with large hydrophobic modifications and thus an increased propensity to form secondary structures in the more lipophilic, single-stranded state was performed next. Even under the seemingly less selective assay conditions of non-self-complementary strands, selection of **21**, the compound known to have the highest affinity for **5**,[21] proceeded smoothly, with the winner in the assay again being discernible from the spectra alone (Figure 9). The protection factor obtained for **21** was higher than that of **4**, the "winner" in the selection from **chemset 1**, in agreement with the greater effect of **21**'s steroid moiety on duplex stability, as seen in melting curve experiments (Table 6).

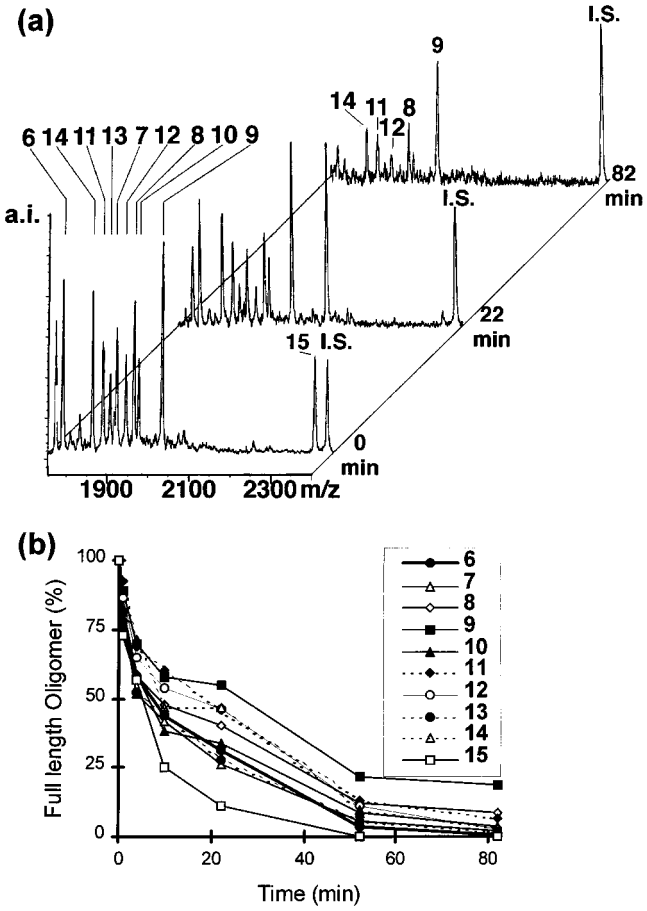


Figure 7. Nuclease selection from **chemset 3**. (a) Selected MALDI-TOF mass spectra; unlabeled peaks in spectra from aliquots taken at 22 and 82 min reaction time are pentamer degradation products. The peak at lowest *m*/*z* is a side product from library synthesis. (b) Kinetics of the nuclease reactions. Data points are averages of relative signal intensities (analyte/internal standard) from four spectra normalized to the signal obtained at t_0 as 100%. Error bars were omitted for clarity.

Table 4. Protection Factors Obtained from Monitored Nuclease Selection from **Chemset 3** at 23 °C

compd	PF[a]	$PF_{co+trumc}$[b]
6	1	1
7	0.96	0.84
8	1.37	1.33
9	1.92	2.20
10	1.12	1.01
11	1.56	1.77
12	1.44	1.64
13	0.98	0.89
14	1.39	1.56
15	0.56	0.49

[a] Protection factor. [b] Protection factor after cutoff and truncation correction with a cutoff of 19% residual full length oligomer.

Based on the few data points currently available, Figure 10 shows the correlations between protection factors (either raw data or corrected for incomplete degradation with "cutoff and truncation") and UV melting points for **chemsets 1**, **2**, **4**, and **5**. For **chemset 3**, this type of analysis will have to await resynthesis and purification of all components. It can be discerned that the nuclease assays not only produced the same qualitative order of target affinity but that, after correction for incomplete degradation, a reasonable correla-

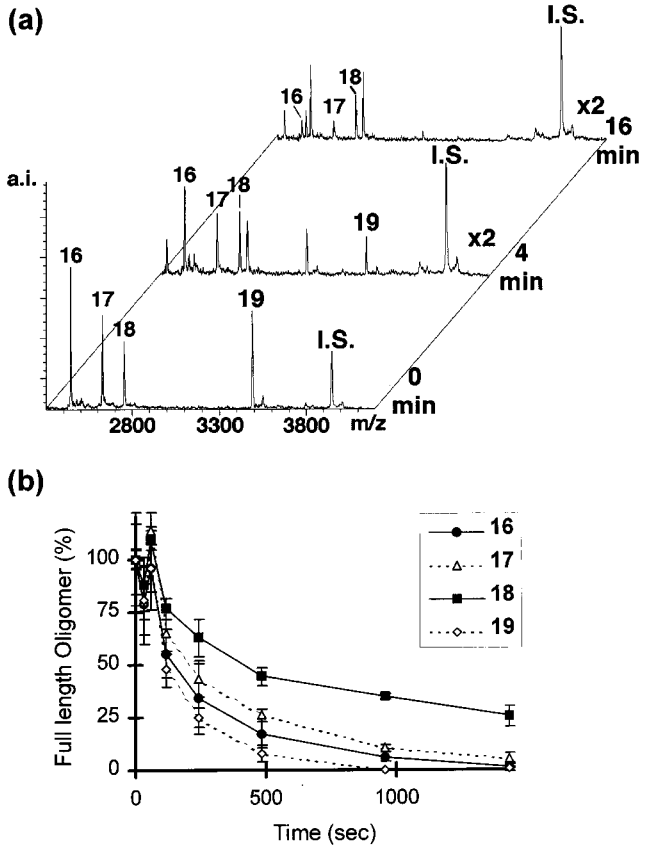


Figure 8. Nuclease selection for high-affinity binders to RNA target **19** in **chemset 4** using snake venom phosphodiesterase. (a) Representative MALDI-TOF mass spectra; unlabeled peaks in spectra from later time points are degradation products from **19** or heptamer products from **16**−**18**. (b) Kinetics of the nuclease reactions. Data points are averages of relative signal intensities (analyte/internal standard) from four spectra normalized to the signal obtained at t_0 as 100%. Error bars are ± one standard deviation; a.i. = arbitrary intensity.

Table 5. Protection Factors Obtained from Monitored Nuclease Selection from **Chemset 4** at 22 °C

compd	target	equivalents complement	PF[a]	$PF_{co+trunc}$[b]
16	**19**	1	1	1
17	**19**	1	1.34	1.42
18	**19**	1	2.31	3.15
19	**16**−**18**	3/0[c]	0.70	0.81

[a] Protection factor. [b] Protection factor after cutoff and truncation correction with a cutoff of 22% residual full length oligomer. [c] Target **19** has three dangling 3′-residues that are vulnerable to nuclease attack.

tion between the values obtained with the traditional single, pure compound assay and our assay with crude combinatorial libraries can be obtained.

Correlation coefficients (r^2 values) are given in Table 7. With the exception of the data for **chemset 2**, the correlation is better for protection factors obtained after the correction for incomplete degradation (column 3 in Table 7). The better correlation of the protection factors with cutoff and truncation correction with T_m data is consistent with the initial phase of the kinetics better representing the equilibrium between bound and unbound modified oligonucleotides. The correlation is further improved when the DNA control compounds are excluded from the analysis (column 3 in Table 7), i.e.,

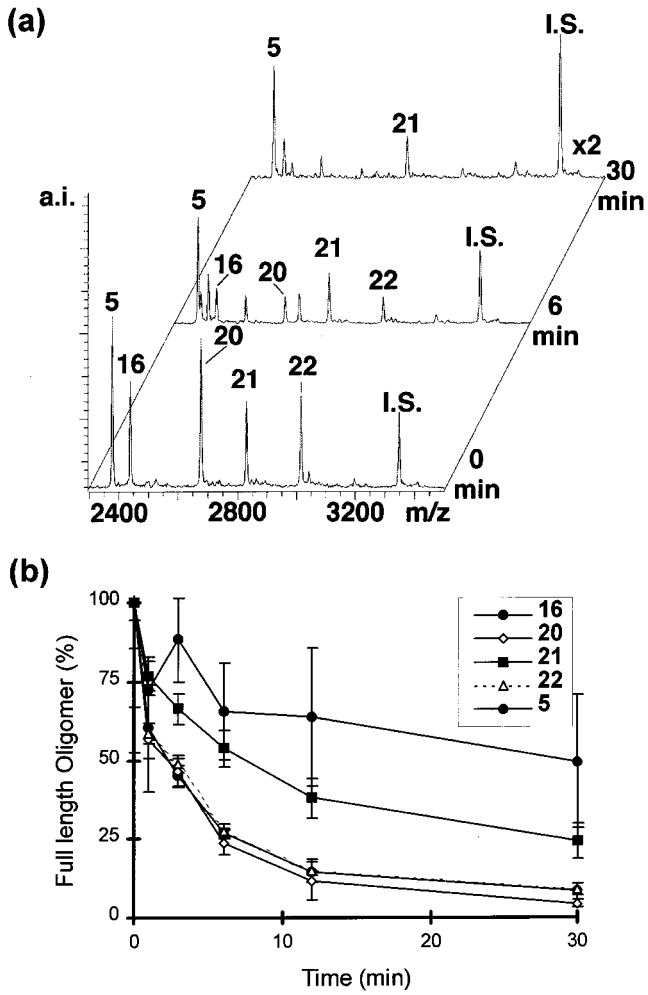


Figure 9. Nuclease selection of compounds in **chemset 5** that bind with increased affinity to DNA target strand **5**. (a) Representative MALDI-TOF mass spectra; unlabeled peaks in spectra from later time points are degradation products. Only **21** and **5** are labeled in the rearmost spectrum for clarity. (b) Kinetics of the nuclease reactions. Data points are averages of relative signal intensities (analyte/internal standard) from four spectra normalized to the signal obtained at t_0 as 100%. Error bars are ± one standard deviation; a.i. = arbitrary intensity.

the oligonucleotide hybrids by themselves cluster better than when plotted with the controls. This effect, which is not just due to fewer data points being correlated, is probably based on the greater structural similarity between the lipophilically modified hybrids. Since the nuclease assays measure kinetics, whereas UV melting curves are acquired under equilibrium conditions, an increase in lipophilic surface area may slow the off-rates more so than it affects the dissociation constant. Even though more data will be needed to generalize any conclusions, the different slopes of the fit functions (Figure 10) indicate that the increase in nuclease protection depends on the duplex used for a selection, its length, RNA or DNA nature, and GC content. Therefore, protection factors can only be translated into predicted melting points after a calibration has been established for a given sequence.

Discussion

The assay reported here is one of several techniques for rapid screening based on mass spectrometry that have been described over the last few years. For example, electrospray

Table 6. Protection Factors Obtained from Monitored Nuclease from **Chemset 5** at 25 °C in the Presence and the Absence of Target Strand **5**

compd	target	equivalents complement	PF[a]	$PF_{co+trunc}$[b]
		With Target Strand		
16	**5**	1	1	1
20	**5**	1	0.87	0.95
21	**5**	1	2.01	3.05
22	**5**	1	1.03	1.06
5	**16**, **20**−**22**	4	3.04	7.69
		Control Experiment Without Target Strand		
16	**5**	0	1	
20	**5**	0	0.72[c]	
21	**5**	0	0.83[c]	
22	**5**	0	0.71[c]	

[a] Protection factor. [b] Protection factor after cutoff and truncation correction with a cutoff of 24% residual full length oligomer. [c] Near-exhaustive degradation of full length oligomer, no cutoff and/or truncation correction; see Supporting Information for plot of kinetics.

mass spectrometry has been used to screen benzene sulfonamide libraries for inhibitors of carbonic anhydrase[31] and to screen carbohydrates for glycosidase inhibition.[32] Neutral loss mass spectrometry of heptapeptide libraries was used to study the substrate specificity of a lantibiotic-synthesizing enzyme,[33] and MALDI-TOF mass spectrometry of peptide ladders was employed in the mapping of protein−protein,[34,35] protein−DNA,[36] and peptide−RNA interactions.[37] For nucleic acids, mass spectrometric analysis of mixtures or libraries has been employed for obtaining sequence information[38−46] or finding sites accessible to ligand binding.[47,48] To the best of our knowledge, this and our previously reported solid-phase-based assay[28] are the only ones that use MALDI mass spectrometry of oligonucleotide mixtures for optimizing the type of chemical modification of oligonucleotides toward increased affinity for a target strand.

All nuclease assays performed show the highest affinity binder as the "survivor", whose relative MALDI signal is more prominent at the end of the assay than those of its competitors. Therefore, a single late data point, together with the spectrum of the initial library, could have sufficed to identify the winner of the competition for the target. A reliable qualitative analysis was available from visual inspection of kinetics with a few data points. Only the need for ranking compounds in multilibrary studies and a correlation with melting points necessitates a protection factor analysis.

The correlation between protection factors and UV melting points (Figure 10) invites a comparison between the two techniques. The nuclease selections presented here have several advantages over synthesizing and purifying single compounds and testing them in UV melting experiments. First, the assay can be performed with crude oligonucleotides, as long as the compound of interest gives a prominent peak in the MALDI mass spectrum. Second, the method is very sensitive. We have used between 120 pmol per compound for pure oligonucleotides and 300 pmol per compound for crude oligonucleotides, i.e., a small fraction of the amount generated in the smallest scale synthesis on commercially available DNA synthesizers. Of the 120−300 pmol used, a small fraction is ionized, allowing optimization toward a "microspot" approach.[49] Since the synthesis of our hybrids is solid-phase based, one may proceed to an integrated synthesis and testing, and ultimately to a "laboratory on a chip".[50] Third, the assay allows the use of combinatorial libraries created through a single synthesis step with mixtures of building blocks, such as the chemset of compounds **11**−**14**. The compounds in the library compete directly against each other in one solution, reducing the experimental uncertainty associated with testing compounds individually, where every experiment has its own pipetting and quantification errors. Finally, since MALDI-TOF MS produces easily predictable spectroscopic signals, the spectra acquired during the spectrometrically monitored assay provide simultaneous quality control for syntheses.[51]

Disadvantages of our technique include the need for approximately 1 h of acquisition time on a MALDI-TOF mass spectrometer per assay, which is more costly than a UV spectrophotometer with melting point capabilities, but less costly than high-field NMR spectrometers used for other spectroscopy-based screening assays.[52] Further, conditions for quantitative detection have to be established. Our results, including those reported in references 27, 28, and 22, indicate that as long as unmodified DNA is structurally dominating, a general protocol can be followed for quite broad a set of modified oligonucleotides, and that as long as linearity is found in a dilution series covering the concentration range of interest, there is no need to purify oligonucleotides for calibration experiments.

The nuclease assays further require a stretch of unmodified DNA or RNA in the binders to be screened to allow the nuclease attack to occur without a bias due to k_{cat} and/or K_M changes for the single strand of the oligonucleotide. Since it is common to test modifications of oligonucleotides with strands containing only a single non-natural residue,[53] this limitation may be tolerable. We have previously shown that degradation of oligonucleotides with nuclease S1 (EC 3.1.30.1), an enzyme that attacks interior phosphodiesters, can also be monitored by quantitative MALDI-TOF MS,[27] allowing for selection from chemsets of oligonucleotides with both 3′- and 5′-modifications. Finally, the change in protection factor when proceeding from **chemset 2** to **chemset 3** may foster concerns that an increase in the size of a chemset is accompanied by loss of selectivity, as seen for screens employing randomized DNA sequences and folded RNA targets.[29] Since repeated assays with **chemset 2** have given us a variation of ca. ±0.3 in protection factors, it is premature to interpret the PF changes observed. Even though the higher affinity hybrids may confer some protection on the lower affinity strands that they bind to in mixed duplexes and may thus potentially reduce the selectivity, the selectivity should not disappear, the way it may with random sequences.[29]

Currently, the nuclease selection assay with self-complementary DNA sequences is about as sensitive in detecting changes in affinity as the traditional UV melting point analysis. Unlike the latter, it can be used for selecting binders to large, folded RNA sequences, where the hyperchromicity associated with the melting of the internal structure of the target dominates melting curves and thus obscures the hyperchromicity of short oligonucleotide ligands. Compared

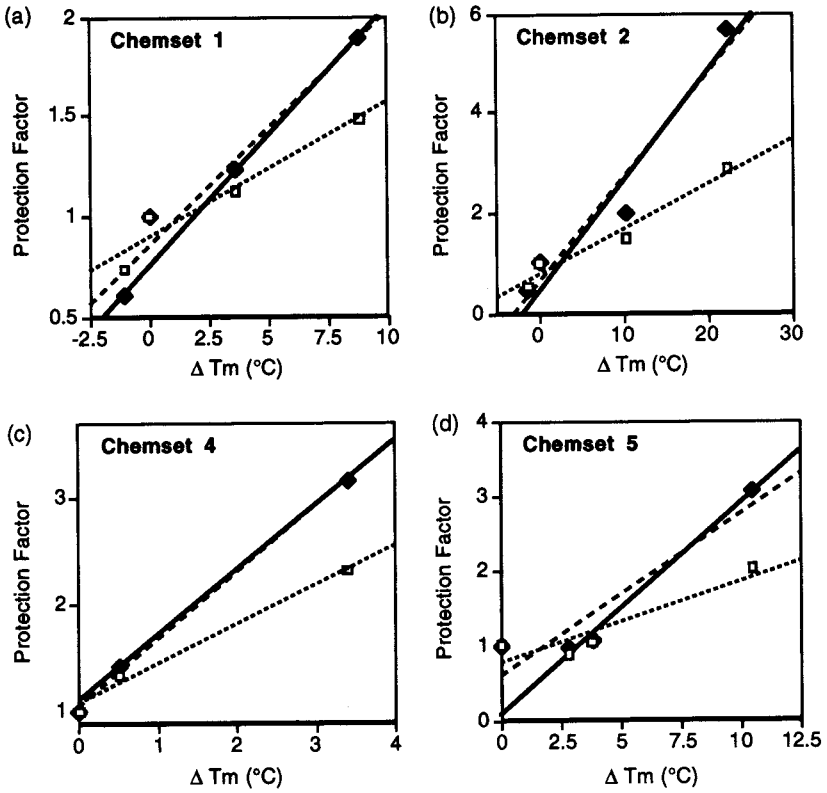


Figure 10. Correlation of UV melting points with protection factors measured in nuclease selection assays. Lines are least square fits. Dotted lines and open squares: correlation with uncorrected protection factors; dashed lines and filled diamonds: correlation with protection factors with "cutoff and truncation" correction for incomplete degradation; solid line: same as dashed line, except that data from the unmodified control sequence were excluded. Melting points were acquired at the following strand concentrations: 1.1 μM (a), 3.7 μM (b), 1.3 μM (c), 1.2 μM (d), and at the following salt concentrations (NH_4OAc): 75 mM (a), 150 mM (b and c), 100 mM (d). See Table 7 for correlation coefficients. Melting points for (a) and (b) are from ref 59, for (c) from ref 19, and for (d) from 21.

Table 7. Correlation Coefficients (r^2) for Linear Fits to UV Melting Point and Protection Factor Data Shown in Figure 10

chemset	PF[a]	$PF_{co+trunc}$[b]	$PF_{co+trunc/wo—co}$[c]
1	0.938	0.958	1.000
2	0.961	0.941	0.945
4	0.987	0.998	*d*
5	0.846	0.877	0.995

[a] Correlation with uncorrected protection factor. [b] Correlation with protection factor corrected for incomplete degradation. [c] Same as *b*, except that the data points of unmodified control compounds were omitted. [d] Trivial, two-point fit.

to SELEX experiments,[13] SMOSE nuclease assays survey a very small number of compounds. The ability to get a full structure-activity map out of the experiments, together with the greater tolerance toward structural modifications in the ligands may outweigh this disadvantage, particularly if genetic algorithms are used for a guided "walk through structure space".[54] If advances in the mass spectrometry of nucleic acids continue,[55−57] current signal-to-noise and molecular weight limitations may be overcome, allowing for mass spectrometrical monitoring of other types of oligonucleotide-based assays, such as analogue interference mapping.[58]

Specific properties of the compounds selected in our assays are in themselves interesting. The 5′-appendages of compounds **4/9**, **17/18**, and **21** strongly increase the affinity of the oligonucleotides they are attached to for complementary strands. Detailed analyses of the binding modes of these hits are being reported separately.[19,21,30,59] From the available two-dimensional NMR data, it has been concluded that the appendages in **17** and **21** stack on the terminal base pair of the Watson−Crick paired duplex without engaging in hydrogen bonding. The stacked appendages bridge both strands of the duplex, thus increasing duplex stability. The strength and specificity of such stacking interactions is difficult to predict, particularly for aliphatic scaffolds, such as steroids. But even among the aromatic acyl groups employed, the difference in melting points between, e.g., **2** and **3** is quite unexpected. The methoxybenzofurane-bearing **3** melts 5 °C higher than the naphthalene-bearing analogue **2**, even though the naphthalene moiety is more lipophilic. This suggests that in the interactions of the appendages with the terminal base pair, enthalpic stacking forces are stronger than the hydrophobic effect, supplementing the conclusions drawn from studies on duplexes with lipophilic dangling residues[60] and extended nucleobase analogues.[61] The lower melting point of the duplex between **2** and **5** compared to that of **1** and **5** can be explained by the absence of the electrostatic stabilization of the duplex by the ammonium group in **1** that is not present in **2**. When compared to noncationic control oligonucleotide **16**, the naphthalene moiety in **2** does not have any stabilizing or destabilizing effect.

Given the subtlety of the stacking and hydrophobic effects, combinatorial synthesis and in vitro selection is a particularly valuable tool for finding minima in the structure space of

terminally modified oligonucleotides. Minima possibly to be found among second-generation oligonucleotide hybrids currently under investigation in these laboratories may create even better "molecular caps" for exposed base pairs. While such exposed base pairs may not be structurally significant in chromosomal DNA, they do exist when DNA is invaded, e.g., by oligonucleotide probes[62] or architectural proteins,[63] turned over by gyrases, or synthesized by polymerases. The latter processes are attractive for therapeutic interventions, and short[64] molecular hybrids based on oligonucleotide leads may become useful drugs in the future.

Conclusion

The results from the exploratory experiments reported here indicate that high-affinity binders to nucleic acid targets can be selected from small chemical libraries of modified oligonucleotides. For these assays, ≤300 pmol of crude oligonucleotide hybrids are needed, and up to 10 compounds can be evaluated in one experiment. Therefore, the search for new oligonucleotide derivatives as ligands for nucleic acid targets may be performed with greater efficiency than with the traditional assays with pure, single compounds. Unlike hybridization assays with oligonucleotide arrays,[65] MALDI-monitored assays have the advantages of allowing selective detection of oligonucleotides without labeling and avoiding the slow kinetics and surface-related effects associated with assays based on DNA chips.[66] The results presented here also show that the terminal base pair of short oligonucleotide duplexes offers an attractive target for ligands employed to modulate duplex stability.

Experimental Section

General. The following chemicals were the best commercially available grade and were used without further purification: DMF, ethanol, and Hünig's base (Fluka), ammonium hydroxide (VWR, EM brand), HOBT, and HBTU (Advanced ChemTech), 2,4,6-trihydroxyacetophenone monohydrate (THAP), diammonium citrate, 1-naphthoic acid, and 7-methoxy-2-benzofurancarboxylic acid (Aldrich), and oxolinic acid (Sigma). Phosphoramidites for dA^{Bz}, dC^{Bz}, and T were obtained from Perseptive Biosystems/Perkin Elmer, dG^{dmf} was from ABI/Perkin Elmer. The protected 5′-amino-5′-deoxythymidine phosphoramidite was prepared as described previously.[15] Controlled pore glass (cpg) preloaded with the 3′-terminal nucleoside was from Perseptive Biosystems/Perkin Elmer. Amino acid building blocks Fmoc-Ala-OH, Fmoc-Pro-OH, Fmoc-Asp(OBn)-OH, Fmoc-Met-OH, and Fmoc-Trp-OH were from Advanced ChemTech, and Fmoc-Lys(Fmoc)-OH was from Novabiochem. All DNA synthesis chemicals were from Perseptive Biosystems/Perkin Elmer, with the exception of the RNA synthesis chemicals obtained from Cruachem USA. Reversed-phase HPLC chromatography was performed with a gradient of acetonitrile in 0.1 M triethylammonium acetate, pH 7, on a 250 × 4.6 mm Alltech Macrosphere 300, 7 μm column (C18), or a 250 × 4 mm Vydac Protein column (C4). The gradients proceeded hyperbolically within the time period given to the maximum acetonitrile content. Yields of hybrids were determined from the integration of the product peaks in the HPLC trace of the crude. The integration was not corrected for the absorbance caused by the solvent front. For lipophilic sequences, recovery from RP18 columns was incomplete, leading to lower yields than achievable with C4 columns or other purification techniques. MALDI-TOF mass spectra were acquired on a Bruker BIFLEX spectrometer with a drift tube extension resulting in 1.85 m path length, a dual microchannel plate detector, a 1 GHz digitizer, and delayed extraction capabilities. Fluences of the N_2 laser (337 nm) are reported according to the manufacturer's specifications and were not measured independently. Spectra were acquired at pressures $<1 \times 10^{-6}$ mbar in the drift tube. Calculated masses are average masses, *m*/*z* found are those for the pseudomolecular ions ($[M - H]^-$), detected as the maximum of the unresolved isotope pattern. The accuracy of mass determination with the external calibration used is ca. ±0.1%, i.e., ±2 Da at *m*/*z* 2000.

General Synthesis Procedure for Oligonucleotide Hybrids. 5′-Aminoacyl-oligonucleotides and 5′-peptidyl-oligonucleotides **10**−**14**, **17**, **18**, and **22** were prepared following a general synthesis protocol previously reported.[15] Given below is a representative procedure for the synthesis of 5′-acylamide-5′-deoxyoligonucleotides **2**−**4**, **7**−**9**, **20**, and **21**. Fully protected oligonucleotides were assembled on an ABI model 381 DNA synthesizer following the manufacturer's recommendation, system software version 1.5, on a 1 μmol scale. The last coupling step was performed with a solution of 5′-*N*-monomethoxytrityl-5′-amino-5′-deoxythymidine-3′-[(2-cyanoethyl)-*N*,*N*-diisopropylphosphoramidite].[15] After drying by exposing the reaction column to 0.1 Torr, a sample of the support (0.17 μmol of oligonucleotide) was transferred into a polypropylene reaction column for ABI DNA synthesizers (empty ABI snap style, 1 μmol size, Prime Synthesis, Aston, PA). The oligonucleotide was detritylated using a deblock step on the DNA synthesizer (1 μmol scale), followed by washing with acetonitrile (10 mL). The support was dried in the column at 0.1 Torr. A mixture of the carboxylic acid to be coupled (100 μmol), HOBT (15.3 mg, 100 μmol), and HBTU (34.1 mg, 90 μmol) was dried at 0.1 Torr for 30 min and dissolved in DMF (600 μL). The solution was treated with diisopropylethylamine (40 μL, 234 μmol), leading to slight darkening. The reaction mixture was introduced into the reaction column containing the oligonucleotide-bearing support with a syringe (1 mL capacity). Excess coupling mixture was taken up in a second syringe attached to the second port of the column. Care was taken to displace all air bubbles from the column. The reaction was allowed to proceed for 60 min with flow-mixing every 5 min. The coupling solution was then pushed out and the glass support washed with CH_3CN (2 × 3 mL), followed by drying at 0.1 Torr. The support was transferred to a polypropylene reaction vessel and treated with concentrated ammonium hydroxide (1 mL) for 16 h at room temperature. The supernatant was transferred into a new vessel, and excess ammonia was removed with a gentle air stream directed onto the surface of the solution for 30 min. For compounds used without purification, the solution was then lyophilized to dryness, relyophilized once from deionized water, and taken up in 25 μL of deionized water. An aliquot (1 μL) was diluted

to 500 μL of water and quantified by UV spectrophotometry at 260 nm. For compounds to be purified, the pH of the solution of the crude product was adjusted to 7.0 with glacial acetic acid, followed by filtration (0.2 μm Whatman PVDF syringe-tip filter) and treatment with ammonium acetate solution (200 μL, 1.0 M, pH 6.0). The resulting solution was used for HPLC purification. Pure fractions were identified by MALDI-TOF mass spectrometry, pooled, dried, and relyophilized once from deionized water. Solutions of oligonucleotide hybrids were quantified by UV spectrophotometry using extinction coefficients calculated as the sum of those of the oligonucleotide and the carboxylic acid.

Chemset 1. (a) Compound 1. HPLC (C4): CH_3CN gradient 0% for 5 min, to 8% in 45 min, R_t 43.7 min. MALDI-TOF MS for $C_{79}H_{100}N_{30}O_{47}P_7$ $[M - H]^-$: calcd 2438.6, found 2437.

(b) Compound 2. HPLC (C4): CH_3CN gradient 0% for 5 min, to 23% in 45 min, R_t 40.7 min. MALDI-TOF MS for $C_{90}H_{107}N_{30}O_{48}P_7$ $[M - H]^-$: calcd 2592.8, found 2593.

(c) Compound 3. HPLC (C4): CH_3CN gradient 0% for 5 min, to 20% in 45 min, R_t 44.5 min. MALDI-TOF MS for $C_{89}H_{107}N_{30}O_{50}P_7$ $[M - H]^-$: calcd 2612.8, found 2612.

(d) Compound 4. HPLC (C4): CH_3CN gradient 0% for 5 min, to 25% in 45 min, R_t 38.2 min. MALDI-TOF MS for $C_{92}H_{109}N_{31}O_{51}P_7$ $[M - H]^-$: calcd 2681.8, found 2681.

Chemset 2. (a) Compound 6. Yield 39%, HPLC (C18): CH_3CN gradient 0% for 5 min, to 17% in 45 min, R_t 43.2 min. MALDI-TOF MS for $C_{58}H_{74}N_{24}O_{33}P_5$ $[M - H]^-$: calcd 1790.2, found 1791.

(b) Compound 7. Yield 49%, HPLC (C18): CH_3CN gradient 0% for 5 min, to 28% in 45 min, R_t 42.3 min. MALDI-TOF MS for $C_{69}H_{80}N_{24}O_{34}P_5$ $[M - H]^-$: calcd 1944.4, found 1946.

(c) Compound 8. Yield 36%, HPLC (C18): CH_3CN gradient 0% for 5 min, to 50% in 40 min, R_t 40.7 min. MALDI-TOF MS for $C_{68}H_{80}N_{24}O_{36}P_5$ $[M - H]^-$: calcd 1964.4, found 1963.

(d) Compound 9. Yield 42%, HPLC (C18): CH_3CN gradient 0% for 5 min, to 50% in 40 min, R_t 30.7 min. MALDI-TOF MS for $C_{71}H_{83}N_{25}O_{37}P_5$ $[M - H]^-$: calcd 2033.4, found 2033.

Chemset 3. (a)Compounds 6−9 are described above (chemset **2**).

(b) Compounds 10−14. A chemset consisting of compounds **10−14** was prepared by coupling a mixture of Fmoc-Ala-OH (3.5 mg, 11.2 μmol), Fmoc-Pro-OH (7.1 mg, 21 μmol), Fmoc-Asp(OBn)-OH (5.1 mg, 11.4 μmol), Fmoc-Met-OH (4.8 mg, 12.9 μmol), and Fmoc-Trp-OH (4.5 mg, 10.3 μmol) with cpg (3.5 mg, 0.11 μmol loading) bearing the 5′-amino-5′-deoxy-terminal and protected DNA sequence TGCGCA using the general synthesis procedure given above. Deprotection with ammonium hydroxide and lyophilization produced a crude chemset that was used without further purification.

(c) Compound 10. MALDI-TOF MS for $C_{69}H_{84}N_{26}O_{34}P_5$ $[M - H]^-$: calcd 1976.4, found 1978.

(d) Compound 11. MALDI-TOF MS for $C_{63}H_{81}N_{25}O_{34}P_5$ $[M - H]^-$: calcd 1887.3, found 1889. Following nuclease selection, compound **11** was resynthesized on 0.1 μmol scale and purified. Yield 53%, HPLC (C18): CH_3CN gradient 0% for 5 min, to 16% in 50 min, R_t 51.4 min.

(e) Compound 12. MALDI-TOF MS for $C_{63}H_{83}N_{25}O_{34}P_5$ $[M - H]^-$: calcd 1921.4, found 1923.

(f) Compound 13. MALDI-TOF MS for $C_{62}H_{78}N_{25}O_{36}P_5$ $[M - H]^-$: calcd 1905.3, found 1907.

(g) Compound 14. MALDI-TOF MS for $C_{61}H_{79}N_{25}O_{34}P_5$ $[M - H]^-$: calcd 1861.3, found 1863.

(h) Compound 15. Oligodeoxyribonucleotide 5′-GTGCG-CAC-3′ was prepared by standard DNA synthesis. Yield 71%, HPLC (C18): CH_3CN gradient 0% for 5 min, to 27% in 50 min, R_t 51.9 min. MALDI-TOF MS for $C_{77}H_{97}N_{31}O_{46}P_7$ $[M - H]^-$: calcd 2409.6, found 2408.

Chemset 4. (a) Compound 16. Yield 50%, HPLC (C18): CH_3CN gradient 0% for 5 min, to 17% in 45 min, R_t 31.4 min. MALDI-TOF MS for $C_{79}H_{99}N_{29}O_{48}P_7$ $[M - H]^-$: calcd 2439.6, found 2439.

(b) Compounds 17−19 have been reported previously,[19] albeit without characterization data.

(c) Compound 17. HPLC (C18): CH_3CN gradient 0% for 5 min, to 17% in 30 min, R_t 31.3 min. MALDI-TOF MS for $C_{90}H_{110}N_{32}O_{48}P_7$ $[M - H]^-$: calcd 2624.8, found 2624.

(d) Compound 18. HPLC (C18): CH_3CN gradient 0% for 5 min, to 18% in 30 min, R_t 28.9 min. MALDI-TOF MS for $C_{96}H_{121}N_{34}O_{49}P_7$ $[M - H]^-$: calcd 2753.0, found 2753.

Chemset 5. Compounds **16** and **20−22** were synthesized individually and purified by HPLC to >95% purity, following the general synthesis protocol given above. Details of the synthesis, characterization, and binding properties of **20−22** are being published separately.[21]

Target Strands. (a) Compound 5. DNA oligomer **5** was synthesized on an ABI model 381 DNA synthesizer following the manufacturer's recommendation (system software version 1.5), it was purified to ≥95% purity by HPLC, and its structure was confirmed by MALDI-TOF mass spectrometry.[21]

(b) Compound 19. RNA undecamer **19** was prepared using 5′-*O*-dimethoxytrityl-2′-*O*-Fpmp phosphoramidite building blocks rA^{Bz}, rG^{iBu}, rC^{Bz}, and U (Cruachem USA). Chain assembly, deprotection, and purification were performed according to the supplier's recommendations (Cruachem Technical Bulletin No. 037R01). HPLC of the Fpmp-protected intermediate was performed on a Vydac 250 × 10 mm C18 protein column in 0.1 M triethylammonium acetate with a CH_3CN gradient from 20% to 55% in 30 min, R_t 19.9 min. MALDI-TOF MS for **19**, for $C_{105}H_{131}N_{44}O_{73}P_{10}$ $[M - H]^-$: calcd 3487.2, found 3488.

Nuclease Selection Assay, General Procedure. The following protocol for the nuclease selection from **chemset 4** is representative. Aliquots (2.5 μL, 1.2×10^{-10} mol) of solutions (4.8×10^{-11} mol/μL) of each oligonucleotide **16**, **17**, **18** were pipetted into a polyethylene reaction vessel (500 μL capacity). To this was added 2.5 μL (1.2×10^{-10} mol) of a solution of *r*(GUCAACCACGG) (**19**), followed by addition of 1.75 μL of 500 mM ammonium acetate buffer in DEPC-treated water. The resulting solution was heated to 90 °C for 1 min, followed by centrifugation at room temperature to recover condensation water. The reaction solution was then cooled to 0 °C and kept at this temperature

for 5 min. The sample was then brought to 23 °C and the $t = 0$ aliquot was withdrawn (1 μL). Then, a solution of phosphodiesterase I (EC 3.1.4.1, Sigma) (1.5 μL of a solution of 1×10^{-4} u/μL) was added, and aliquots of 1 μL were taken at the desired time points. The aliquots withdrawn from the reaction mixture were immediately mixed thoroughly with 5 μL of the matrix mixture. The matrix mixture had been made up from 100 μL of 2,4,6-trihydroxyacetophenone solution (0.3 M in ethanol), 40 μL of diammonium citrate solution (0.1 M in water), 60 μL of acetonitrile, and 3.9 μL of a solution of the internal standard 5′-AGCTCAGT-TAGCT-3′ (4.08×10^{-10} mol), resulting in 1.02×10^{-11} mol of internal standard in each 5 μL matrix aliquot. Immediately after the mixing, 1.5 μL of the solution of analytes and matrix was applied to a stainless steel MALDI target plate. The plate had earlier been prepared by coating with silicone oil, followed by removal of excess oil by wiping with wipes soaked in ethanol and ammonium hydroxide. The matrix/analyte mixture was left drying at room temperature, and residual water was evaporated with a hair dryer and then the prechamber vacuum of the mass spectrometer. Spectra were acquired in negative, linear mode at 20 kV with a delayed extraction voltage of 18.2 kV and 180 ns delays for laser shots at 60−70 μJ/shot. A total of 100 shots at 2 Hz laser frequency was accumulated for every spectrum. At least four spectra were acquired per time point. Analyte and internal standard peaks were integrated, and the relative intensities of the analyte peaks were calculated and expressed as fractions of the $t = 0$ min value. Spectra with less than 500 ion counts for the internal standard peak were not used for data analysis.

Selection from Chemset 1. Nuclease selections from **chemset 1** were performed as described above for **chemset 4** with the following modifications: a solution of oligonucleotides **1**−**4** (300 pmol of each crude oligonucleotide, based on quantitation with extinction coefficient of pure compounds) and the chosen amount of target strand **5** (150, 300, or 900 pmol, 0.5, 1, or 3 equiv) in 11.75 μL of DEPC-treated water 75 mM in ammonium acetate, pH 6, was prepared. The solution was heated to 55 °C for 10 min, allowed to cool to room temperature, and brought to the desired temperature (10, 23, 30, or 40 °C) with a water bath. An aliquot of 3.2 μL of the nuclease solution (10^{-4} u/μL) was used. Aliquots from the assay mixture (1 μL) were mixed with 3 μL of a matrix solution made up from a solution of THAP matrix (100 μL, 0.3 M in EtOH), a solution of diammonium citrate (40 μL, 0.1 M in water), acetonitrile (60 μL), and a solution of the DNA oligomer 5′-CGCATT-AGCA-3′ (15 μL, 92 μM in water) as internal standard. A total of 200 laser shots per spectrum were acquired in this case.

Selection from Chemset 2 and Chemset 3. The nuclease selections from **chemsets 2** and **3** were performed at 23 °C as described for **chemset 1**, above, with solutions of crude oligonucleotides. The concentrations of the stock solutions used for preparing the assay solutions were also determined spectrophotometrically assuming solutions of pure compounds. MALDI spectra of all crude compounds showed ≥75% of the desired product by peak integration of the *m*/*z* region 1000−4000. For these assays, DNA oligomer 5′-TGGTTGAC-3′ (**16**) was used as internal standard.

Selection from Chemset 5. The selection of high-affinity compounds from **chemset 5** was performed as described above for **chemsets 1**−**3** with a solution (15 μL total, 83 mM in ammonium acetate) containing HPLC-purified oligonucleotides **5**, **16**, and **20**−**22** (125 pmol each), heated to 76 °C for 2 min, cooled to 0 °C, brought to 25 °C, and treated with 1.0 μL of nuclease solution (10^{-4} u/μL). Aliquots of 1.5 μL of the assay solution were mixed with 5 μL of the matrix solution prepared as for selections from **chemset 1**, except that the DNA oligomer 5′-CCGTGGTTGAC-3′ was used as internal standard.

Data Analysis. MALDI spectra were analyzed in automated fashion using two computer scripts, DAS 1.0 and AUTOMATON 1.1. The code for both programs will be made available for downloading via the Internet from the Richert Research Group Homepage (currently http://microvirus.chem.tufts.edu). Macro DAS 1.0 was written in AURA and runs under Bruker XTOF, version 3.1, on a SUN SPARC 5 with Sun OS release 5.5 generic. DAS reads MALDI spectra, expands to a chosen *m*/*z* region, rescales the *y*-axis, applies two-point smoothing and a five-point filter to the raw data, integrates predefined *m*/*z* regions corresponding to the peaks of interest, and saves and prints peak and integral lists. AUTOMATON, written in PERL and running under the system specifications given above, extracts report files created by DAS, compiles them into lists corresponding to a given time point in the assay, and selects four spectra with the greatest signal intensity in every list, using the ion counts for the internal standard as a criterion. AUTOMATON then calculates the average of the normalized peak intensity, ratios of analytes and internal standard in the selected spectra, together with the standard deviation. Negative integrals, encountered at full degradation of an analyte and a negative slope in the baseline, are set to zero. AUTOMATON then connects data points for every analyte and calculates the area under this point-to-point fit. After normalizing the area for the control oligonucleotide, protection factors are calculated as ratios of analyte versus control areas. To allow for correction for incomplete degradation of one or several analytes at the end of the assay, cutoff and truncation corrections to the areas and the resulting protection factors can be calculated with AUTOMATON, as graphically explained in Figure S1 of the Supporting Information. In this work, the cutoff was chosen as the percent full length oligonucleotide of the least degraded chemset component at the end of the assay. AUTOMATON has a capability for calculating the statistical significance of protection factors, using the standard deviations of the data points of analyte and control oligonucleotide and error propagation for area and protection factor calculations. The final output can be directly imported into a spreadsheet (as tab delimited text) and printed with any common graphing software.

UV Melting Points. UV melting curves were measured on a Perkin-Elmer Lambda 10 spectrophotometer equipped with a Peltier-thermostated six-cell holder driven by a PTP-6 temperature unit and a temperature sensor in cuvette 1. Melting curves were measured at 260 nm between 5 and 75

°C at a heating or cooling rate of 1 °C/min. Melting points were determined using Perkin Elmer UV TempLab 1.2. Curves were smoothed with a 95-point moving average, followed by calculation of the 91-point first derivative, whose extremum gave the melting point (Tm). At least four individual curves were acquired per duplex, and reported values are the average of the melting points.

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Supporting Information Available. A graphical definition of protection factors, MALDI-TOF mass spectra of all oligonucleotide hybrids, first derivatives of melting curves shown in Figure 2, and the kinetics of a nuclease run with **chemset 5** without target strand. This material is available free of charge via the Internet at http://pubs.acs.org.

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