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Destabilizing Universal Linkers for Signal Amplification in Self-Ligating Probes for RNA

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Abstract: Recent studies have established the utility of oligonucleotide ligation methods in the detection of DNAs and RNAs in solution and in cellular imaging. Notably, the ligated full-length oligonucleotide products commonly bind to the target nucleic acid much more tightly than do the two starting half-probes, which effectively limits the resulting signals to one per target. Here, we report on a molecular strategy for destabilizing ligated products in template-promoted self-ligation reactions, thus yielding multiple signals per target. A new universal linker design is described in which a dabsyl leaving group is placed on a short alkane tether. This allows the placement of an electrophile at the end of any DNA sequence, in contrast to earlier ligation strategies, and it also speeds reaction rates by a factor of 4−5. This new class of molecular linker/activator yields as much as 92-fold amplification of signals in DNA and RNA detection, and proceeds without enzymes, added reagents, or thermal cycling. The linker is shown to destabilize the ligation product without destabilizing the transition state for ligation. This lowers product inhibition, and the target DNA or RNA thus becomes a catalyst for isothermally generating multiple signals for its detection. This enhanced signal generation is demonstrated in solution experiments and in solid supported assays.

Introduction

Because many genetic samples exist in small amounts, methods for amplification of signals in detection have been widely investigated. Enzymatic approaches involving polymerases, such as PCR,[1−3] or other enzymes such as thermostable ligases,[4−6] have been quite useful in this regard. In a new strategy, a number of laboratories have begun to investigate the use of nonenzymatic fluorescence-based approaches for RNA and DNA detection that rely on the formation of bonds,[7] hybridization of fluorescent oligonucleotides,[8,9] or changing secondary structure[10,11] to detect genetic sequences. Nonenzymatic approaches offer possible advantages in cost, simplicity, and application in cells where needed enzymes are not present. However, such nonenzymatic strategies have not yet been shown to yield appreciable amplification of signals, which would clearly be helpful in detecting RNAs or DNAs at low concentrations or in low numbers.

Recent studies have reported the application of self-ligation reactions in RNA and DNA sequence detection.[12−15] In such approaches, the chemistry for the joining of two short oligonucleotide probes is incorporated into the ends of the molecules themselves;[16] a DNA-joining reaction is simple in that it not only requires no enzymes, but also needs no added reagents beyond the DNA probes themselves. In a recent advance, the chemistry for ligation was activated by a group (dabsylate) that acted both as a leaving group and as a fluorescence quencher, thus enabling the probes to become fluorescent in the presence of a complementary target.[14] Such self-ligation reactions have been shown to be highly selective for single nucleotide differences in the target molecule. The absence of a requirement for enzymes or reagents has enabled these quenched probes to be applied in cellular RNA sequence detection.[17,18]

Although such self-ligation reactions have shown considerable promise, they also display some limitations as well. One is that the rate of reaction is significantly slower than enzymatic ligations, in some cases requiring hours rather than minutes.[13,14] Second, the DNA end activation was placed directly on a terminal T nucleoside; thus, this approach would require the synthesis of four different modified nucleosides (some of them chemically unstable) for application to all sequences in general.[14] Finally, ligation reactions (both enzymatic and nonenzymatic)

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typically yield only one signal per target molecule under isothermal conditions, thus presenting limited detectability when the target is in low concentration.

Here, we report on a new molecular strategy that yields improvements in all three of these areas. This approach uses a new class of universal dabsyl-quenched linkers to promote ligation with nucleophilic DNA probes. Unlike previous approaches, the quenched linkers allow ready application to any DNA sequence and are added in an automated step on a DNA synthesizer. Surprisingly, the rates of ligation with these linkers are found to be significantly higher than previous ligation activating strategies. Finally, the linkers are shown to be strongly destabilizing to hybridization once the linkage is made, and as a result they promote turnover of probes on the target, offering up to 100-fold amplification of the fluorescence signal without enzymes or thermal cycling.

Materials and Methods

Synthesis of Dabsyl Linker Phosphoramidite Derivatives. Details of the synthesis and characterization of universal linker phosphoramidites (compounds **3a**, **3b**) are given in the Supporting Information.

Oligonucleotide Probes. Oligodeoxynucleotides were synthesized with β-cyanoethylphosphoroamidite chemistry and were purified by PAGE gels. Dabsyl and fluorescein-modified oligonucleotides were prepared following literature procedures and were characterized by mass spectrometry. Details are available in the Supporting Information.

Dabsyl-Mediated Autoligation Reactions. Ligations were performed in 3 mL of pH 7.0 PIPES (70 mM) buffer containing 10 mM $MgCl_2$, 50 μM dithiothreitol with target nucleic acid (100 nM), 7mer 3′-phosphorothioate probe (100 nM), and dabsyl-labeled 8mer probes (100 nM, respectively) at 25 °C for 2 h. Reactions were observed by fluorescence spectrometry (Fluorolog 3-11, Jobin Yvon-SPEX). To observe the kinetics of the ligation reactions, fluorescence intensity was measured with 5 s integration at 1 min intervals: excitation was at 494 nm, and the fluorescence of FAM was measured at 518 nm. Reactions using radiolabeled probes were incubated at the indicated temperatures and times. Samples were heated to 95 °C for 2 min and loaded on 20% polyacrylamide gel containing 8 M urea. Radioactivity on gels was quantitated on a Molecular Dynamics phosphorimager (Amersham).

Reactions on PEG-polystyrene beads were carried out using 3′-phosphorothioate oligonucleotide immobilized on PEG-polystyrene beads. They were incubated in 50 μL of pH 7.0 PIPES (70 mM) buffer containing 10 mM $MgCl_2$ and 50 μM dithiothreitol with 50mer target DNA (1 nM) and dabsyl-labeled 8mer probes (10 μM) at 30 °C for 24 h. See the Supporting Information for details.

Results and Discussion

The Principle of Product Destabilization in Probe Design. The molecular approach used here for detection of genetic sequences relies on the principle of the self-directed reaction of two oligonucleotide probes bound side-by-side on a target strand of DNA or RNA (Figure 1). The reaction of the nucleophilic phosphorothioate group on one probe with the 5′ electrophilic carbon of the adjacent probe causes displacement of a dabsylate group, which acts both as a fluorescence quencher and as a leaving group.[14] This results in the formation of a bond between the two probes, yielding a double-stranded segment as long as the combination of the two starting probes. Because a fluorescent label is also present on the electrophilic DNA probe, the loss of the dabsylate quencher results in an increase in fluorescence as probes become ligated. Because the probes are short, their binding to mismatched targets is weak, allowing

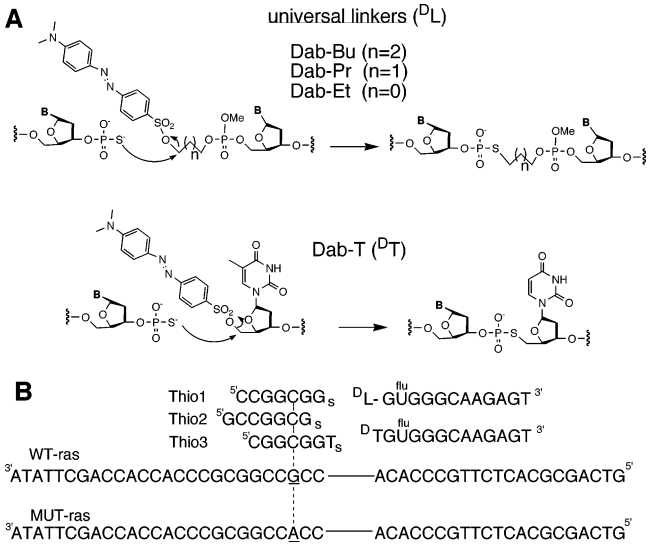

Figure 1. Structures and sequences studied. (A) Structures and ligation mechanism of three new dabsyl-quenched universal linkers reported herein, as compared to the previous dabsyl-dT for self-ligating DNA probes. (B) DNA sequences tested for ligation reactions. Thio1−Thio3 are nucleophilic probes containing 3′-phosphorothioate groups (denoted by subscript "s"); "flu" denotes fluorescein-conjugated T.

the method to give high selectivity for single nucleotide differences.[13,19]

Previous designs placed a dabsylate group directly on the 5′ hydroxyl of the electrophile oligonucleotide probe (Figure 1, Dab-T).[14] The ligation reaction then resulted in a linkage between the two probes that was very nearly the same as that in natural DNA, the only difference being that one bridging oxygen was replaced by sulfur. This small difference causes little or no destabilization to the DNA helix.[20] Thus, the ligation results in two weakly binding probes being converted to a longer tightly binding oligonucleotide, which is strongly inhibited from dissociating from the target DNA. For example, if one compares binding of complementary DNA by two previously studied 7mer + 13mer half-probes to that of the expected 20mer ligated product, the T_m is calculated to rise from ca. 60 to 85 °C, and binding affinity is expected to become much more favorable, by at least several kcal/mol.[21] In practical terms, this limits the signal formed per target molecule to not much greater than one under isothermal conditions. Although technically the target might act as a catalyst for ligation of probes, the catalysis suffers from strong product inhibition.

The new molecular design presented here offers a strategy for overcoming this product inhibition, by selectively destabilizing the ligated product as compared to the previous sulfur-bridged linkage (Figure 1A). In this new approach, the electrophilic dabsylate group is placed at the end of a hydrocarbon linker chain, which is in turn linked to the 5′ end of the electrophile probe via phosphoramidite chemistry (Figure 1A; universal linkers). This 5′ linker is not expected to cause strong changes in binding by this oligonucleotide prior to reaction. Models suggest also that a nucleophile probe should be able to bind adjacent (coaxial) to the electrophile probe with little difficulty. As a result, one might expect this linker to make little difference to the energy of the starting probe/target

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complex. After ligation, the situation is different, however: the ligated probe now contains a several-atom-length flexible linker interrupting the two complementary half-segments that are complementary to adjacent positions on the target. This complex is expected to be considerably less favorable entropically than having a direct linkage between the two half-probes. For example, abasic linkers are known to destabilize DNA markedly.[22,23] Thus, the ligated probe/target complex is expected to dissociate much more readily than in the previous directly linked approach, even under isothermal conditions. This dissociation would, in principle, allow a new pair of probes to bind, generating multiple signals per target.

Linker Probe Design. To test this, we constructed a series of three linkers of varying length, based on ethylene glycol (DEt), 1,3-propanediol (DPr), and 1,4-butanediol (DBu) (see Figure 1A). These are expected to change the energy of the ligated product complex and also possibly change the rate at which ligation occurs, by offering varied distances and geometries relative to the phosphorothioate nucleophile. Dabsylate was placed at one end of each diol linker, and an *O*-methyl phosphoramidite was placed at the other. The *O*-methyl group was chosen because early studies with the standard cyanoethyl group suggested that the phosphate anion (generated after deprotection) could react intramolecularly with the arylsulfonate function,[24] generating background signals even in the absence of target (data not shown). The *O*-methyl group was found to strongly diminish this background reactivity. To preserve the potentially labile methyl triester, we used mild deprotection conditions and employed Pac-protected phosphoramidites in the probe synthesis.[25,26]

For comparison, we also tested probes designed to generate bulges with the target. Bulged nucleotides also are known to destabilize segments of perfectly complementary double helices,[27] and so we designed different bulge geometries to test: a case with an extra nucleotide in the probe strand, and one with an extra nucleotide in the target (Figure 1B). Finally, we also tested for comparison a pair of perfectly complementary probes with the previously described 5′-dabsylate group situated directly on the 5′-hydroxyl (abbreviated DT).[14] That class of probe generated only ca. one signal per target at moderate target concentrations, although small amounts of turnover were previously observed with a similar iodide-activated case at low target concentrations.[13]

Ligation Rates. Ligation reactions were carried out in PIPES buffer (pH = 7) containing 10 mM $MgCl_2$. Relative rates of reaction were compared at equimolar target and probe concentrations (100 nM each), and the progress was followed in solution by the increase in fluorescence signal. The results are shown in Figure 2. The data show that, under these conditions, all three new linkers (DEt, DPr, and DBu) gave ligation that was more rapid than that for the previous dabsyl-thymidine case (DT) in Figure 2A. The most rapid was the DBu case (Figure 1), which ligated at a rate ca. 4-fold higher than the previous directly linked 5′-dabsyl approach (DT),[14] based on initial slopes of the curves. The DPr case was ca. 3-fold faster, while the DEt case was faster than the DT by a small factor of ~1.2. This increase in rates was initially surprising, as models would suggest that the linkers might position the electrophilic carbon further from the nucleophile. A possible explanation for this is that the geometry in the DT case is suboptimum for the displacement reaction and that the flexibility of the new linkers increases the likelihood of reaching a favorable geometry.

We also evaluated the ligation rates for the bulge-geometry strategies with the previous standard, the DT probe (Figure 2B). Here, the data clearly showed that either a bulge in the probes (DT + thio3) or a bulge in the template (DT + thio2) slowed the rate considerably, by a factor of 3.2. Both bulge geometries yielded similar rates. This result is less surprising, as one might expect the extra nucleotide either to block the ability to reach a favorable geometry for reaction or to add unfavorable free energy at the transition state by disrupting base local base pairing or stacking.

In a third experiment, we examined the effect of combining different nucleophile probes with the DPr linker case. This was done because with the new linkers it is not clear whether a linker should be considered as a proxy for a nucleotide or not (note that the three-carbon chain is the same number of carbons as a deoxyribose residue in a DNA backbone). When we compared the relative rates for the three cases (Figure 2C), we observed that the case where the linker is considered extraneous to the pairing (DPr + thio3) was by far the fastest at ligation. It showed a better than 7-fold advantage in rate as compared to the other two alignments (DPr + thio1, DPr + thio2). This result, combined with the above bulged nucleotide experiments, suggests the importance of coaxial stacking of the two DNA probes in helping to form complexes that are productive for ligation.

Finally, we compared the rates for ligation with the new linkers when the target is complementary or mismatched by a single nucleotide. In this experiment, the mismatch (C/A) was situated at the center of the heptameric nucleophile probe binding site. The results showed (Figure 2D) that the rate was indeed sensitive to a single mismatch, with a 12.3-fold drop in ligation rate with the DBu linker on the mismatched target, and a 9-fold drop with the DPr linker. This order-of-magnitude selectivity is comparable to sequence selectivities observed for other methods,[10] although it is smaller than was reported previously for 5′-iodide-mediated autoligating probes.[13] The origin of this difference is unclear, but may arise from differences of analytical methods between gel electrophoresis (in which background is subtracted) and fluorescence spectrometer (which includes background).

Measurement of Turnover. Turnover, and the associated signal amplification, is useful when target concentrations are low. Under these conditions, one would typically use a large excess of probes relative to the numbers of targets. To measure turnover with the current probes, we compared the number of equivalents of signal to moles of target. This required the development of a method for carefully quantitating the signal, and confirming that the signal arises from true template-dependent intermolecular ligation reactions rather than from background sources such as (i) incomplete quenching of fluorescence by dabsyl; (ii) hydrolysis or other release of

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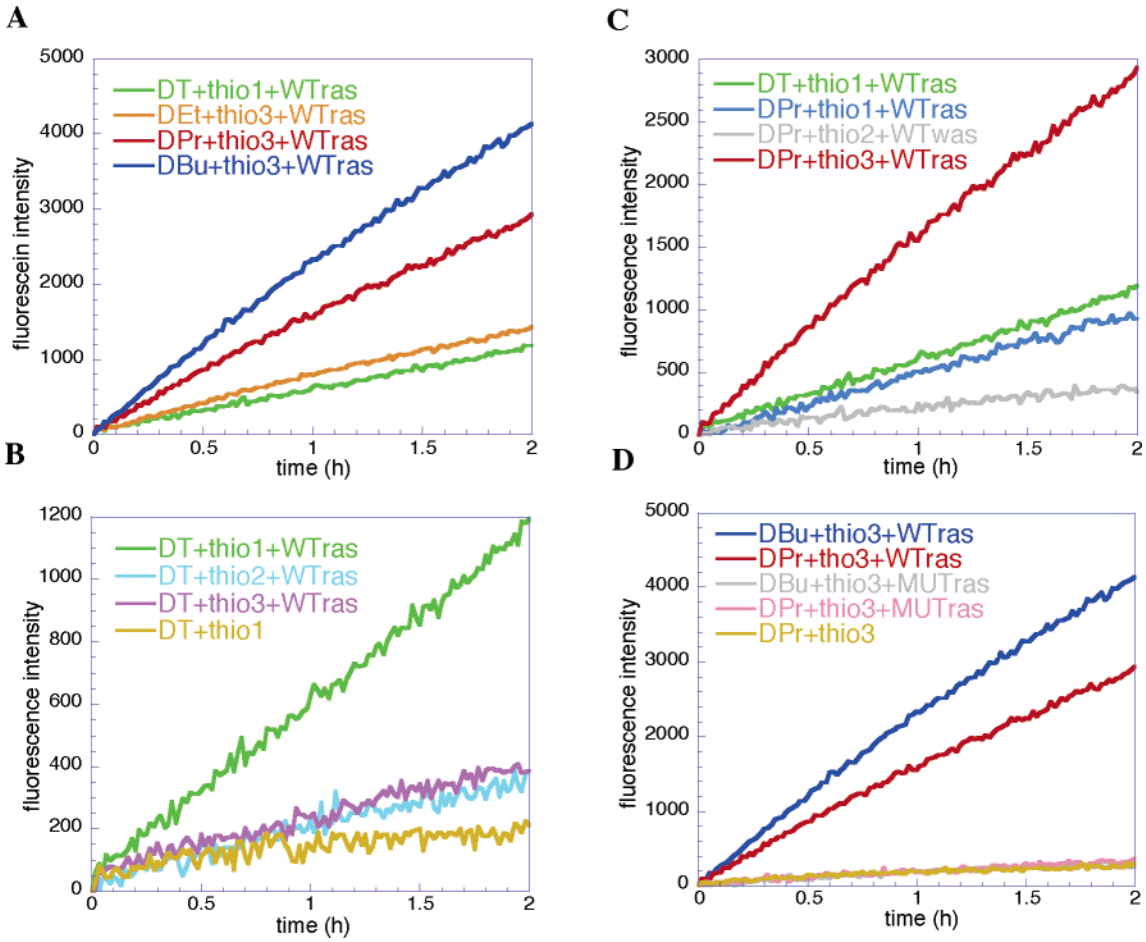

Figure 2. Effect of linker structures and sequences on ligation rates, as measured by fluorescence intensity over time. (A) Comparison of electrophilic linkers DEt, DPr, and DBu to previous dabsyl-T (DT) strategy. (B) Rates of bulged nucleotide strategies (see Figure 1). (C) Effect of nucleophile location on rates of DPr linker ligations. The dabsyl-T (DT) case is shown for comparison. (D) Effects of single-base mismatches on relative ligation rates for DPr and DBu universal linkers. The reactions were done under the following conditions: dabsyl probe (100 nM), phosphorothioate (100 nM), target DNA (100 nM) in pH 7.0 PIPES buffer (70 mM) containing $MgCl_2$ (10 mM) at 25 °C.

dabsylate from the electrophile probe; or (iii) intermolecular ligation independent of the template strand. To evaluate this, we performed ligations using radiolabeled phosphorothioate probes at varied target concentrations and temperatures (see below), and we separated products from excess unreacted probes by gel electrophoresis. An image of such a gel is shown in the Supporting Information (Figure S1). The radioactivity in the ligated product bands was quantitated by digital phosphorimaging. We first prepared a standard dilution curve to make a calibration plot of radioactivity as a function of the amount of ^{32}P-labeled phosphorothioate oligonucleotide in a gel lane. This plot showed good linearity and allowed us to take a given signal and, from the plot, extract the number of moles of radioactive oligonucleotide product in a given band on the gel.

Effect of Target Concentration on Amplification. The turnover of ligated products from the target RNA or DNA is expected to increase as the concentration of target decreases. To test this, we varied target concentrations over the range from 1000 nM to 1 nM. The butanediol-type universal linker probes were used for turnover measurement because this linker yielded the most rapid ligations. The probe concentrations were held constant at a considerable excess (10 μM), and the reactions were evaluated at 25 °C after relatively long incubations of 24 h. To ensure that signal was not the result of template-

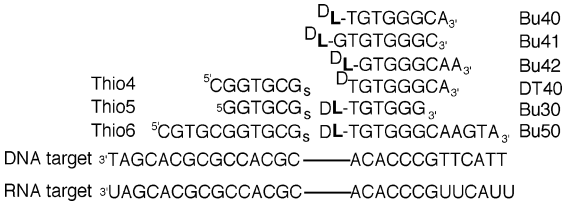

Figure 3. Probe and target sequences studied for turnover experiments. Thio5 and Thio6 are short and longer variants of Thio4. DL refers to the dabsyl-conjugated butanediol universal linker.

independent ligations or hydrolysis, we subtracted background signals from identical reactions lacking templates (see Figure S1).

The target concentration effect was measured for universal butanediol (DBu) linkers as well as for the original dabsyl-T electrophile (Figure 3). The position of the 8mer butanediol linker probe on the template was shifted relative to the 7mer phosphorothioate probe, yielding ligated products with different bulged complementarity (Bu40, Bu41, Bu42). The results showed (Figure 4) that for all four cases significant amounts of turnover were observed, and for all four there was a marked increase in turnover with lowering target concentration. At the lowest concentration, the number of turnovers was least for the DT40, with maximum turnovers of ca. 8-fold at the 1 nM target concentration. However, the universal linker cases showed

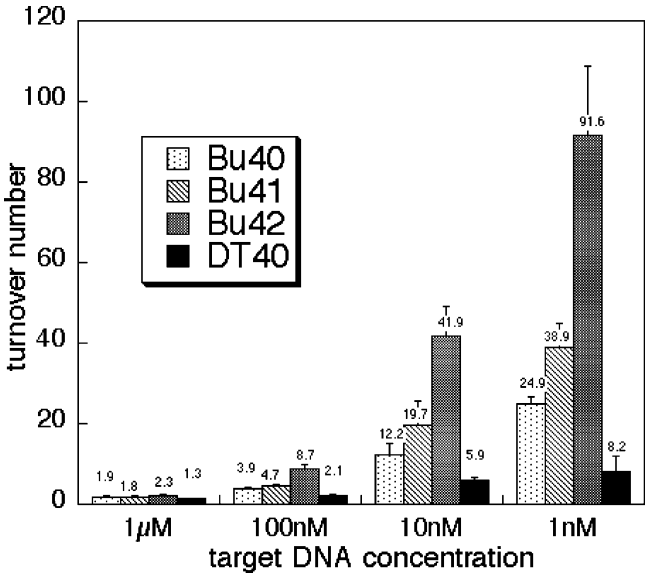

Figure 4. Effect of target DNA concentration on turnover for four electrophilic probe designs. Turnover is given as equivalents of radioactive signal per mole of target DNA. The concentration of target DNA was varied at 1000, 100, 10, and 1 nM, respectively, in a pH 7.0 buffer (70 mM Tris-borate buffer) containing 10 μM dabsyl probe (Bu40, Bu41, Bu42, or DT40), 10 μM phosphorothioate probe (thio4), and 10 mM $MgCl_2$ at 25 °C. Reactions were analyzed by 20% polyacrylamide gel electrophoresis after 24 h. Reactions were run at least three times, and the data were averaged; error bars show standard deviations.

considerably higher turnover numbers. The coaxial case (Bu40, where the linker is extraneous to the sequence) was the lowest, but still yielded up to 25 equiv of signal per target; the case in which probes skip a base (Bu42, yielding a bulge in the target strand) was most efficient of all, yielding 92 turnovers. The case in which the probes are overlapped (Bu41, yielding a 1-nt bulge in the probes) fell between the two, at 39 turnovers. Thus, we conclude that, of all of the new molecular strategies, the Bu42 case yields the most efficient turnover under these conditions.

Effect of Temperature. The temperature is expected to have significant effects both on ligation rates and on turnover efficiency. Ligation chemistry may be expected to increase in rate with temperature; however, template binding by the unligated probes is expected to begin to melt at higher temperatures, which would remove the template effect and slow the rate again. As a result, one expects an intermediate temperature range, perhaps near the T_m of the shorter probe, where a maximum in rate is reached.

To test for such effects and optimize turnover, we carried out ligations in which we varied temperature over the range 15−45 °C in 10 °C increments (Supporting Information, Figure S2). As before, all four ligation chemistries were compared. For most cases, we observed an increase in turnover efficiency with increasing temperature, going through a maximum, then dropping at the highest temperatures (see the Supporting Information). The original DT40 probes gave very little turnover at any temperature. Once again, the case in which the probes yield a bulge in the target strand (Bu42) proved to be most effective, giving as high as 92 turnovers at optimum temperature, 25 °C. For most cases, maximum turnover was seen at 25 °C and least turnover was seen at 45 °C.

Effect of Probe Length. The probe length is also expected to have significant effects both on ligation rates and on turnover

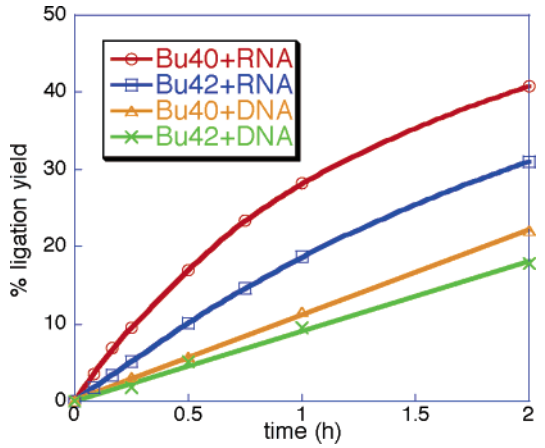

Figure 5. Comparison of ligation rates with RNA versus DNA templates. Ligations were carried out under the following conditions: linker probe, 1 μM; thio4, 1 μM; RNA or DNA template, 1 μM at 25 °C.

efficiency. Lengthening probes should result in more significant product inhibition, as dissociation would be greatly slowed. On the other hand, longer probes should rapidly ligate based on strong hybridization. To test this, we designed a short probe pair and a long probe pair, which yield ligation product with bulge structure (Bu30/thio-5 and Bu50/thio-6, respectively) (Figure 3). The ligation rate with the 12-mer probe Bu50 was 30-fold greater than the 6-mer probe Bu30, and 1.3-fold greater than the 7-mer (Supporting Information, Figure S3). However, the longest and shortest probes gave lower turnover number (6−7) than the 7mer in 24 h. Thus, we conclude that probes that are too short or too long are unfavorable for turnover, due to slow ligation or strong product inhibition, respectively.

Testing Ligation on an RNA Target. Because RNA−DNA duplexes form different helical structure than do DNA−DNA duplexes, there is no guarantee that such ligation chemistry would proceed the same in detecting RNA as compared to DNA. Thus, we prepared an RNA target and compared reaction rates to the previous DNA target. Interestingly, ligation rates on the RNA template were more rapid than the corresponding DNA template (Figure 5). Using initial rates, the Bu40 and Bu42 cases were faster than those with the DNA target by factors of 2.9 and 3.2, respectively. The turnover number of Bu42 on the RNA template was 78 at 25 °C for 24 h, similar in magnitude to the number on the DNA template.

Analysis of Destabilization by the Alkanediol Linkers. Our initial hypothesis was that turnover in this self-ligation reaction would be favored when the ligated product does not bind well to the template. To evaluate this, we isolated ligated 15mer products, for the original dabsyl-T linkage from DT40 (shown here to turn over poorly) and the butanediol-linked case Bu42 (the most effective turnover case). We measured binding of these products to a target DNA by thermal denaturation. The results (Figure 6) showed that the melting temperature of combined unligated probe pairs for the two cases was essentially the same (45 °C); however, the ligated product from Bu42 (T_m = 58.5 °C) was much more destabilized with target DNA than was the product from DT50 (T_m = 70.7 °C). This adds support to our product destabilization hypothesis and suggests that efforts aimed at even greater product destabilizations may be warranted in the future.

Self-Ligation and Turnover on Solid Support. Bead-supported and glass-supported genetic detection methods are

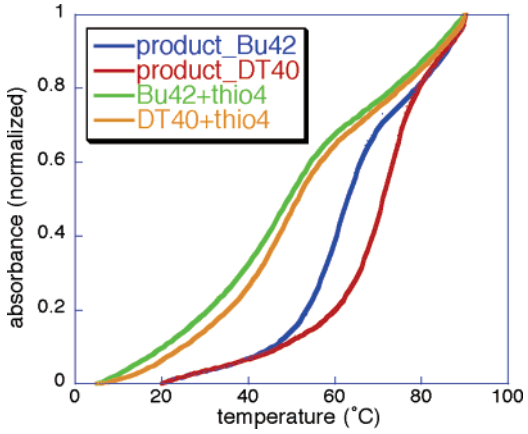

Figure 6. Thermal stability of unligated probes and ligated products, showing destabilization by universal linker. Ligated products from Bu42 and DT40 were isolated and tested for binding of target DNA. Conditions: 2.5 μM oligonucleotide, 10 mM $MgCl_2$ in pH 7.0 PIPES buffer (70 mM). Absorbance changes were normalized.

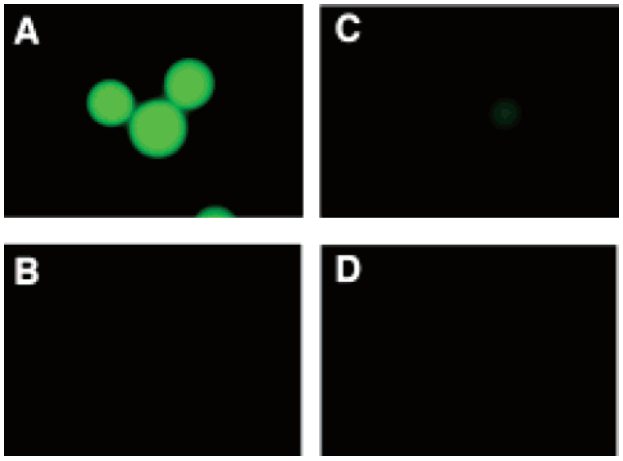

Figure 7. Signal amplification during DNA detection on solid support. Ligation reactions were done on phosphorothioate-conjugated (CGGTGCGs) beads using 10 μM butanediol linker probe Bu42 (A) with 1 nM target or (B) without target DNA, or 10 μM DT40 probe (C) with 1 nM target or (D) without target DNA at 30 °C for 24 h. No washing to remove unreacted probes was done after ligations.

now widespread.[28−30] We have previously demonstrated that under nonturnover conditions, a bead-supported nucleophile probe could ligate dabsyl-quenched probes to themselves in the presence of template DNA.[18] This was done with dabsyl-T electrophiles, which the present results showed to be poor at turnover. It would potentially be of considerable utility in some applications if turnover were also possible on the beads or arrays: one template could, in principle, be passed from probe to probe on the bead surface, again generating multiple signals per equivalent of template and amplifying signals that might otherwise be weak.

To test this possibility, we prepared PEG-polystyrene beads with a phosphorothioate-derivatized oligonucleotide probe with its 3′ terminus free (by application of reverse 5′ → 3′ oligonucleotide synthesis). We reacted these beads in the presence of target DNA, with either the original dabsyl-T probe or the butanediol-universal-linked dabsyl probe, which was shown (above) to be most proficient at turnover. Figure 7 shows beads after 24 h of incubation with Bu42 (A) and DT40 (C). The beads treated with Bu42 were brighter than those treated with DT40 by a factor of 17. Little signal was seen from beads without target DNA (Figure 7B,D). In addition, background fluorescence remained low; thus, no washing of the beads was required, making the method exceedingly simple. Thus, we conclude that the ability to undergo multiple turnovers results in a marked difference of signal intensity on beads with the new universal linker.

Implications in Probe Design and Application. The new linkers, particularly the butanediol- and propanediol-based quenched linkers, offer significant advantages over previous self-ligating probe chemistries. These two optimum cases offer more rapid rates than previous quencher-activated probes. For example, under equimolar probe-target conditions, the butanediol case reaches 30% yield within 120 min, as compared to 12 h with the dabsyl-T activation. Under excess probe (turnover) conditions, the butanediol linker offers 10-fold signal amplification in as little as 160 min, and >90-fold in 24 h. Importantly, RNA detection was even more rapid than DNA detection, a result that is markedly different from enzymatic ligations, where RNA is a poor target.[31]

It is also worth noting that the new linkers are quite simple and inexpensive to prepare, and they can be appended to any DNA oligonucleotide in automated steps on a standard DNA synthesizer. In addition, the hydrophobicity of the dabsyl group offers ease of purification by reverse-phase HPLC, in analogy to the widely used "trityl on" purification strategy. A previous limitation of the earlier dabsyl-mediated ligations was the requirement for a 5′-thymine on the electrophile probe, which resulted in some sequence limitations.[14] The new approach can be applied universally to any probe sequence, and thus any target site. This ease of preparation and application increases the utility of the self-ligating probe strategy.

In addition to the ease of synthesis and enhanced reaction rate, we expect that the turnover observed for the new probes may offer significant utility in detection/identification of target RNAs and DNAs when the copy number or concentration is low. Because we have not yet determined whether there is a useful linear range for the amplification as a function of time, the chief applications of turnover would initially be qualitative target sequence identification rather than, for example, RNA quantitation. One of our chief goals is qualitative identification and imaging of RNAs in cells,[18] which we hope to identify by color at single nucleotide resolution. Work is underway to apply the universal quenched linker strategy to that purpose.

A few laboratories have recently investigated strategies for using pairs of modified oligonucleotides to generate amplified products or signals. In a novel approach that does not require ligation, Taylor[32,33] and Kraemer[34] have described the combination of a hydrolysis catalyst on one oligonucleotide with a leaving group (in the form of an ester) on the other, resulting in the release of multiple leaving groups for each targeted

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complementary strand of DNA. Those approaches have generated ca. 3−35 turnovers. Further, the former has reported the generation of fluorescence signals albeit without the demonstration of turnover.[35] Ligations of amino-conjugated oligonucleotides have been investigated by von Kiedrowski[36] and by Lynn.[37] The former approach requires denaturation cycles for turnover (unlike the present approach). The latter strategy isothermally generates as much as >50 turnovers in ligation, but it requires a separate reagent (borohydride), and it is not clear how the approach could generate easily detectable signals. Seitz has recently developed peptide nucleic acid (PNA) probes that ligate by native chemical ligation;[15] such probes have not yet been demonstrated to undergo turnover, nor do they generate fluorescent signals. Finally, RNA-detecting ribozymes are well documented to undergo turnover;[38,39] however, a recent example by Sando of a DNAzyme designed to generate fluorescence signals in detection of an RNA documented only four signals per target.[40] In contrast to these approaches, the current strategy is isothermal and yet undergoes significantly more turnovers (yielding an amplification of nearly 2 orders of magnitude). Also, unlike most of those previous strategies, it readily generates easily detectable fluorescence signals and can even be used in a multicolor format.[18] Ongoing work will be aimed at testing the new universal linkers in the detection of cellular RNAs.

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Supporting Information Available: Experimental details of modified probe synthesis and ligation methods. This material is available free of charge via the Internet at http://pubs.acs.org.

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