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A Two-Component Small Molecule System for Activity-Based Detection and Signal Amplification: Application to the Visual Detection of Threshold Levels of Pd(II)

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Supporting Information

ABSTRACT: A detection and signal amplification strategy aimed toward threshold diagnostic assays for use in resource-limited settings is described. The strategy employs two small molecule reagents that work in tandem. One reagent detects a specific analyte, while the second amplifies a colorimetric readout autocatalytically. The strategy is demonstrated using palladium(II) as a model analyte.

Diagnostic assays are a crucial component of medical care in resource-limited settings, yet the reagents used in most point-of-care assays lack the thermal stability needed for environments encountered in the developing world.[1] Currently, the most sensitive and selective diagnostic assays rely on antibodies, enzymes, silver(I) salts, or a combination of these reagents to achieve sensitivity and selectivity. These reagents, however, are not stable for prolonged periods when stored above 0 °C. To circumvent this limitation, point-of-care diagnostics typically contain additives and/or are refrigerated to help maintain the activity of the reagents. Even with these measures in place, sensitive and selective assays like enzyme-linked immunosorbent assays (ELISA),[2] PCR,[3] biobarcode assays,[4] and other similar assays are not easily translated to resource-limited environments.[1] Given the dearth of temperature-stable reagents, other analyte detection and signal amplification reagents must be developed. This deficiency also provides the opportunity to develop basic science that leads to new, general strategies for detection and signal amplification.

A handful of research groups have described small molecule reagents that avoid, to some extent, the thermal instability problems described above.[5] Each of these seminal efforts has provided a reagent that specifically detects a single analyte and amplifies the resulting signal. For example, Anslyn and Koide both have developed catalytic systems for detecting Pd and Pd^{2+}.[6] Anslyn reported two additional signal amplification systems: one for detecting Cu^{2+}, and the other for Pb^{2+}.[5,6] Mirkin described a supramolecular system for detecting the combination of Cl^- and CO,[7] as well as another reagent that responds to the combination of acetate and CO,[7] and Shabat recently reported a reagent for detecting thiols.[8] Polymer-based reagents for detection and signal amplification also have been described; examples include Swager's versatile conjugated polymers for fluorescent and/or electrical readouts[9] and the degradable dendrimer reagents developed by McGrath, de Groot, and Shabat.[10]

Despite these advances, there remains a need for a general small molecule (or polymer) detection and amplification system that can be used to detect a variety of analytes in resource-limited environments. Ideally, the system should be readily accessible, thermally stable, specific in its response to an analyte, and compatible with complex biological fluids (i.e., not susceptible to background reactions). Furthermore, the system should (i) amplify the signal quickly (minutes to hours), (ii) amplify the signal to levels that rival or exceed standard analytical methods (e.g., ELISA assays or PCR), (iii) provide a clear visual readout that does not require spectrophotometers or electronics for detection, (iv) be easily reconfigurable to detect trace levels of different analytes, and (v) provide qualitative and quantitative readouts.

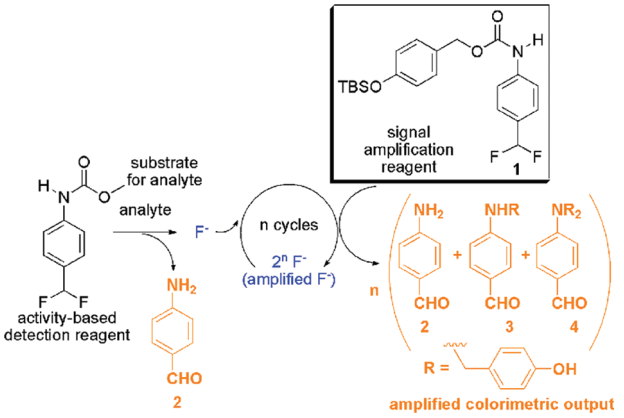

Figure 1. Design of a thermally stable, two-component, small molecule system for detection and signal amplification. The detection reagent reacts specifically with an analyte and releases a signal transduction reagent (e.g., F^-). The signal amplification reagent then reacts with this signal transduction reagent in an autocatalytic process to produce amplified quantities of a colorimetric indicator (compounds **2**–**4**).

This communication describes a new detection and signal amplification strategy that we hope will provide the starting point for realizing these goals. Our strategy (Figure 1) employs two thermally stable small molecule reagents that work in tandem to provide selective, trace-level detection of specific analytes. The first reagent in this pair is an activity-based detection reagent that reacts with the analyte (e.g., Pd(II)) and releases a specific chemical signal (e.g., fluoride). The signal amplification reagent then responds to this released signal (fluoride) in an autocatalytic degradation reaction that releases amplified quantities of a yellow indicator (e.g., compounds **2**–**4**, discussed below) until the

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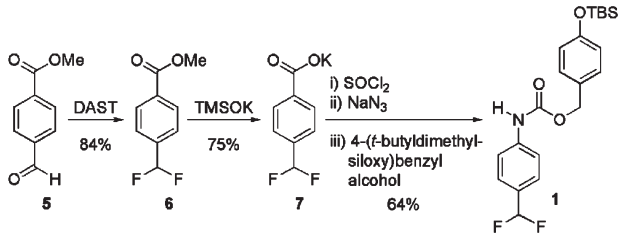
Scheme 1. Synthesis of Amplification Reagent 1



amplification reagent (**1**) is consumed. **2**−**4** can be detected visually, thus enabling detection of threshold levels of an analyte.

Because two separate reagents control the detection and amplification processes, this system can be easily reconfigured to detect and amplify a variety of analytes, which is a feature that is not common in other single molecule detection and signal amplification reagents. Using our design, the amplification reagent can be used in any assay, while the reactive portion on the activity-based detection reagent (i.e., "substrate for analyte" in Figure 1) can be modified to detect a variety of analytes.

To evaluate the efficacy of this detection and amplification strategy, we first prepared and studied the amplification reagent (compound **1**). Reagent **1** was obtained via an efficient (40% overall yield), three-step synthesis (Scheme 1).

The amplification process using **1** is initiated by fluoride-induced cleavage of the *tert*-butyldimethylsilyl-protected phenol on reagent **1**, which results in the formation of quinone methide and azaquinone methide. In addition, two new equivalents of fluoride are released, and these ions react with additional equivalents of **1** to propagate the amplification reaction. As this iterative process continues, the quantity of fluoride at the end of each cycle, in theory, will be equal to 2^n, where n is the number of cycles. As the amplification reaction progresses, increasing numbers of indicators **2**−**4** are released, thus strengthening the colorimetric readout.

We tested the behavior of **1** by dissolving it in MeOH and pyridine[11] and then exposing it to a stoichiometric amount of fluoride (CsF) dissolved in water (final solution: [**1**] = 0.12 M in 18:1:2 MeOH/pyridine/H_2O, 105 μL). Under these conditions, **1** is consumed completely within 120 min. The quinone methide intermediate arising from fluoride-induced decomposition of **1** reacts with 4-aminobenzaldehyde (**2**, the product arising from release of fluoride) to produce colored byproducts **3** and **4** (Figure 1).

The use of stoichiometric fluoride makes the autoinductive properties of **1** difficult to observe. However, when **1** is exposed to a substoichiometric quantity of CsF, a plot of fluoride concentration versus time produces a sigmoidal curve (Figure 2), as would be expected for an autoinductive or autocatalytic process.[12] We measured the concentration of fluoride arising from the amplification reaction by exposing the amplification solution to excess (relative to the maximum quantity of fluoride expected in the solution) *tert*-butyldimethylsilyl (TBS)-protected 7-hydroxycoumarin. Fluoride reacts with TBS-protected 7-hydroxycoumarin ~60× faster than with **1**. Under these conditions, the fluoride is consumed rapidly, and 7-hydroxycoumarin, a fluorescent indicator molecule, is produced.

In the absence of applied fluoride (Figure 2, dark blue data), **1** is stable for 48 h in MeOH/pyridine/H_2O (18:1:2) at 23 °C. After 48 h, a slow breakdown leads to the release of fluoride, initiating the autoinductive amplification reaction (Figure 2).

To determine the source of the slow background reaction with **1**, we prepared three control reagents (Figure 3) and exposed

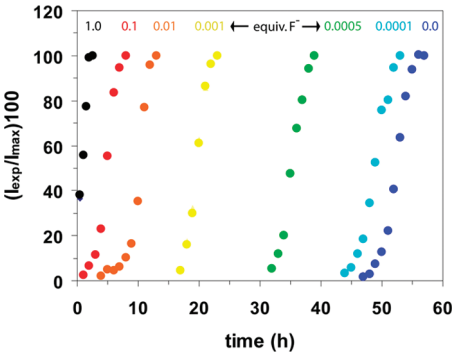


Figure 2. Signal transduction reagent (fluoride) is amplified as amplification reagent **1** is consumed. The graph shows the sigmoidal increase in the concentration of fluoride (expressed in terms of fluorescence intensity (I) of 7-hydroxycoumarin) as **1** is consumed. I_{max} is the maximum fluorescent signal (obtained upon complete consumption of **1**), and I_{exp} is the measured fluorescent signal at any time point in the amplification reaction. Each line corresponds to a different number of equivalents of added fluoride relative to **1**. The experiments using 1.0, 0.1, 0.001, 0.0001, and 0 equiv of fluoride were repeated three times, and those using 0.01 and 0.0005 equiv were repeated twice; the averages of each experiment are shown on the graph. The amplification reactions were conducted at 23 °C in MeOH/pyridine/H_2O (18:1:2, 105 μL); the concentration of **1** in the starting solution was 0.12 M.

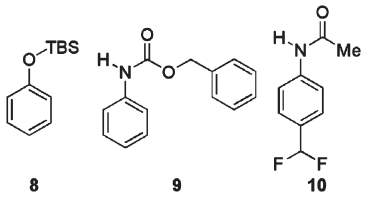


Figure 3. Structures of the control compounds that were used to test the cause of the background reaction shown in Figure 2.

them to the conditions used for amplification (i.e., 0.12 M concentration of each control reagent in 18:1:2 MeOH/pyridine/H_2O, 105 μL, 23 °C). After 21 d, HPLC traces revealed that none of the control reagents showed signs of decomposition (at least within the detection limits of the HPLC).[13] Thus, we hypothesized that perhaps the background signal shown in Figure 2 is the result of trace quantities of adventitious fluoride either in **1** or in our solvents. Trace amounts of fluoride initially would lead to slow, autocatalytic decomposition of **1** until the quantity of fluoride in solution is sufficient to increase the rate of the autocatalytic reaction to a measurable level. However, because the background reaction is slow, even trace levels of fluoride arising from a detection event often will be sufficient to overcome the background reaction.

Reagent **1** is capable of amplifying fluoride to a concentration much higher than the quantity of fluoride added to the system. The amplification factor (α) for fluoride is described by $(I_{amplification} - I_{background})/I_{initial}$, where $I_{amplification}$ is the intensity of the fluorescent signal produced by reaction with TBS-protected 7-hydroxycoumarin after amplification, $I_{initial}$ is the intensity without amplification, and $I_{background}$ is the signal arising from spontaneous breakdown of amplification reagent **1** ($I_{background}$ is only included in the calculation when 0.0001 equiv of F^- is used, as shown in Figure 2). We measured the amplification factor for five concentrations of added fluoride. Figure S2 shows that as the quantity of applied fluoride decreases the amplification factor

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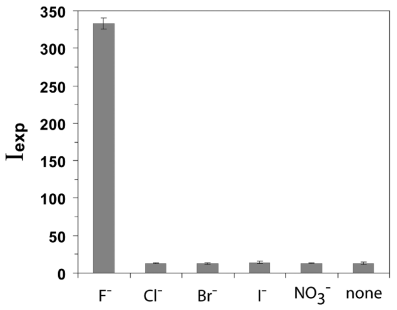

Figure 4. Stability of amplification reagent **1** in the presence of representative anions. In separate experiments, aqueous solutions of CsF, NaCl, NaBr, NaI, and $NaNO_3$ (0.5 equiv) were added to a solution of **1** in MeOH and pyridine. The final concentration of **1** was 0.12 M in 18:1:2 MeOH/pyridine/H_2O (105 μL), and the experiment was conducted at 23 °C. Fluorescence measurements (I_{exp}) representing the quantity of released fluoride were obtained after 4 h of amplification using the procedure described in Scheme S1. Experiments were performed in triplicate, and error bars reflect the standard deviations from the average values. The fluorescence measurements were obtained by treating each sample with TBS-protected 7-hydroxycoumarin. TBS-protected 7-hydroxycoumarin is weakly fluorescent, which gives rise to the same amount of background signal seen with the control, Cl^-, Br^-, I^-, and NO_3^-.

increases rapidly to a maximum value of 2168 ± 35 for 0.0005 equiv of added F^-. This value of amplified fluoride corresponds to ~11 cycles of the autoinductive amplification reaction shown in Figure 1.

Because specificity is critical in the context of our amplification reaction, we examined whether amplification reagent **1** would react with anions other than fluoride (Figure 4). Phenolic silyl ethers are known to be quite unreactive to anions other than fluoride (such as Cl^-, Br^-, I^-, NO_3^-, SO_4^{2-}, SCN^-, and PO_4^{3-}),[14] but we were concerned about the stability of the carbamate and the benzylic difluoro-substituted carbon in **1**. As shown in Figure 4, representative anions such as Cl^-, Br^-, I^-, and NO_3^- have no effect on **1**, thus supporting the notion that **1** may be useful in the context of complex fluids. Furthermore, **1** is stable in air at 37 °C for at least one month with no decomposition or change in reactivity (one month was the duration of our test). In certain parts of the developing world, extreme temperatures may surpass 37 °C, but the stability of the compound in these preliminary tests suggests that this type of amplification reagent may provide a suitable starting point for further development.

We next turned our attention to developing a complete diagnostic system (Figure 1), and we chose Pd(II) as a model analyte. Palladium is capable of cleaving allyloxycarbonyl (Alloc) protecting groups catalytically, even in water; therefore, this diagnostic system serves as a model of more advanced systems that will be used to detect enzymes (experiments to link the amplification reagent with an enzymatic detection event are in progress). Moreover, palladium is an interesting analyte in its own right: it is an environmental contaminant found in roadside soil and on roadside plants, with quantities often exceeding 0.3 ppm due to loss from catalytic converters.[15] Palladium also can be found within some pharmaceutical products (the government-regulated threshold for palladium contamination in drugs is 10 ppm).[16] A handful of fluorescent and colorimetric sensors have been developed recently for detecting palladium,[17] with sensitivities reaching ~0.5 ppm palladium for colorimetric responses.[6b]

To demonstrate the diagnostic system outlined in Figure 1, we first prepared an activity-based detection reagent for Pd(II) (i.e., reagent **11**, Figure 5a) that releases 2 equiv of fluoride for every

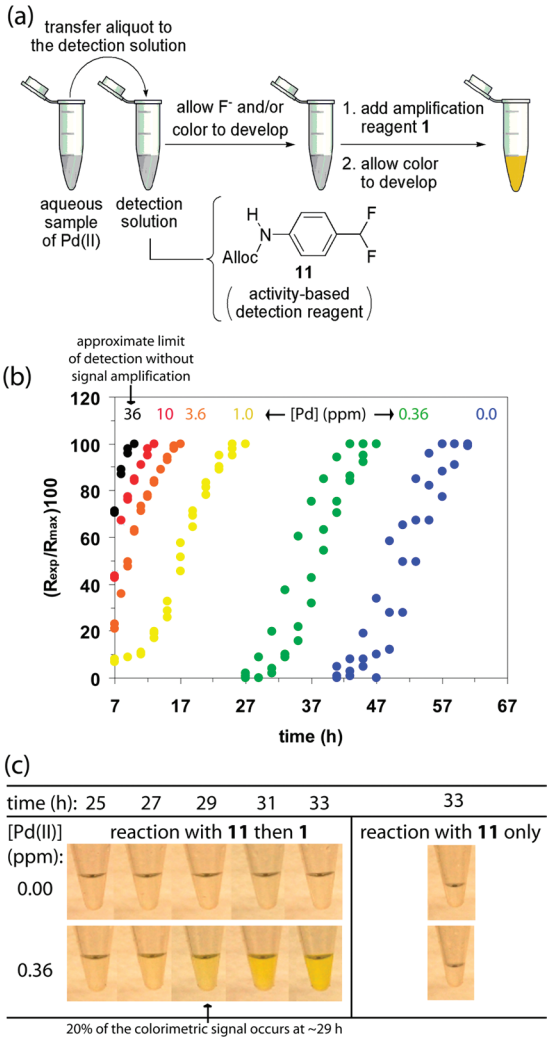

Figure 5. Detection of Pd(II) using a two-component reagent combination. (a) Schematic of the procedure for detecting Pd(II). (b) Reflectance (R) (obtained using a camera and image processing software) of the colorimetric output as a function of assay time. Total assay time equals the time required for activity-based detection (6 h) plus the time required for signal amplification. The y-axis reflects the colorimetric response (R_{exp}), which is reported as the percentage of the maximum possible colorimetric response (R_{max}). Experiments were performed in triplicate; all data are shown on the graph. (c) Photographs of colorimetric response observed with amplification (reaction with **11** and **1**) and without amplification (reaction with **11** only) and in the presence and absence of Pd(II).

catalytic reaction with Pd(0) (via reduction of Pd(II) in situ), as well as 1 equiv of the colored byproduct **2**.

When activity-based detection reagent **11** (1.0 mg in MeOH/pyridine (20:1, 52.5 μL) and 400 μM tri-(2-furyl)phosphine) is exposed to a solution of 36 ppm Pd(II) ($NaPdCl_4$) in water (10 μL) at 23 °C, the colorimetric response reaches a maximum signal within 2 h (as determined by photographing the samples and quantifying the reflectance using imaging processing software). When 3.6 ppm Pd(II) is used, a colorimetric signal does not develop, even after 33 h of incubation (Figure 5c). At such low concentrations of Pd(II), activity-based detection is not sufficiently sensitive to provide a colorimetric readout in this system, and therefore, the activity-based detection reagent must be coupled with a signal amplification reaction.

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This two-component system requires two steps: (i) detection of the Pd(II) to create a fluoride signal, and (ii) amplification of the fluoride with concomitant formation of colorimetric products. Initial experiments were performed with 10 ppm Pd(II) (the upper limit of palladium that is allowed in drugs).[16] When activity-based detection reagent **11** is exposed to Pd(II), the quantity of fluoride released from the reagent increases linearly with time (Scheme S2). After 6 h of incubation,[18] we diluted the detection solution with amplification reagent **1** (42.5 μL, 0.29 M in 16:1 MeOH/pyridine) and followed the development of colorimetric products **2**−**4**. The intensity of the yellow color produced by **2**−**4** was quantified using reflectance measurements via photographs and image processing software. Figure 5b shows the results of this combined detection and signal amplification reaction. The *x*-axis in Figure 5b reflects the total time for the assay (i.e., the time required for detection and amplification), and the *y*-axis reflects the percentage of colorimetric signal observed relative to the maximum colorimetric signal possible under the current reaction conditions.

While the current detection and signal amplification reagents provide signal slowly (ideal assays would require <1 h to obtain results), this detection system is quite sensitive: Figure 5b reveals that even 0.36 ppm palladium (ca. the quantity of palladium found in roadside soil)[15] can be detected by visual inspection. This level of sensitivity compares well with the sensitivity of other colorimetric sensors for palladium.[6b]

Yellow color is apparent at only 20% of the maximum colorimetric signal (i.e., $(R_{exp}/R_{max}) \times 100 = 20$ in Figure 5b) (see Figure 5c for the actual colors), so a threshold level of palladium in a sample can be determined easily by eye. For example, Figure 5c shows that concentrations of palladium that are ≥0.36 ppm cause the solution to turn yellow within 29 h of initiating the detection/amplification reaction. The time is shorter for higher concentrations of Pd(II): e.g., if ≥10 ppm is present in a sample, then the solution turns yellow within 6.5 h.

The strategy outlined has several features making it a starting point for further development of point-of-care diagnostic assays. The results of the detection and amplification process are colorimetric, so they are visually easy to read for qualitative yes/no assays, the reagents are capable of detecting and signaling the presence of analytes that originate in aqueous solutions, and in theory, the activity-based detection reagent can be modified so that it responds specifically to analytes other than Pd(II), which would enable the detection of a variety of analytes using the same diagnostic strategy.

The current detection and amplification system does have some drawbacks; e.g., the strategy requires the use of organic solvents (to dilute the aqueous sample) and pipettes (to supply the sample), and the amplification reaction is slow. Our current efforts are focused on overcoming these issues.

The key component of our new strategy is the signal amplification reagent that produces a colorimetric readout through an autocatalytic process. The proof-of-concept systems described in this communication represent a new approach to designing nonenzymatic, thermally stable, easily accessible, and analyte-specific amplification reagents that may be of broad use in the context of point-of-care diagnostic assays.

■ ASSOCIATED CONTENT

Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ REFERENCES

(1) Giljohann, D. A.; Mirkin, C. A. *Nature* **2009**, *462*, 461–464.
(2) Gosling, J. P. *Clin. Chem.* **1990**, *36*, 1408–1427.
(3) Blow, N. *Nat. Methods* **2007**, *4*, 869–875.
(4) Nam, J. M.; Thaxton, C. S.; Mirkin, C. A. *Science* **2003**, *301*, 1884–1886.
(5) (a) Zhu, L.; Anslyn, E. V. *Angew. Chem., Int. Ed.* **2006**, *45*, 1190–1196. (b) Cho, D.-G.; Sessler, J. L. *Chem. Soc. Rev.* **2009**, *38*, 1647–1662.
(6) (a) Wu, Q.; Anslyn, E. V. *J. Am. Chem. Soc.* **2004**, *126*, 14682–14683. (b) Houk, R. J. T.; Wallace, K. J.; Hewage, H. S.; Anslyn, E. V. *Tetrahedron* **2008**, *64*, 8271–8278. (c) Song, F.; Garner, A. L.; Koide, K. *J. Am. Chem. Soc.* **2007**, *129*, 12354–12355. (d) Garner, A. L.; Koide, K. *Chem. Commun.* **2009**, 86–88. (e) Garner, A. L.; Koide, K. *J. Am. Chem. Soc.* **2008**, *130*, 16472–16473. (f) Garner, A. L.; Song, F.; Koide, K. *J. Am. Chem. Soc.* **2009**, *131*, 5163–5171.
(7) (a) Yoon, H. J.; Mirkin, C. A. *J. Am. Chem. Soc.* **2008**, *130*, 11590–11591. (b) Masar, M. S., III; Gianneschi, N. C.; Oliveri, C. G.; Stern, C. L.; Nguyen, S. T.; Mirkin, C. A. *J. Am. Chem. Soc.* **2007**, *129*, 10149–10158.
(8) Sella, E.; Weinstain, R.; Erez, R.; Burns, N. Z.; Baran, P. S.; Shabat, D. *Chem. Commun.* **2010**, *46*, 6575–6577.
(9) Thomas, S. W., III; Joly, G. D.; Swager, T. M. *Chem. Rev.* **2007**, *107*, 1339–1386.
(10) Avital-Shmilovici, M.; Shabat, D. *Soft Matter* **2010**, *6*, 1073–1080.
(11) This solvent system was chosen after a screen of other solvents. Reagent **1** is not soluble in pure water, and therefore, we explored mixtures of water and MeCN, DMF, DMSO, MeOH, and THF. We chose the combination of solvents that gave the fastest signal amplification reaction. The relative order of reactivity was MeOH > DMSO > DMF > MeCN > THF. We included pyridine as a precaution to buffer any HF that might develop during the signal amplification process.
(12) (a) Tjivikua, T.; Ballester, P.; Rebek, J., Jr. *J. Am. Chem. Soc.* **1990**, *112*, 1249–1250. (b) Severin, K.; Lee, D. H.; Martinez, J. A.; Vieth, M.; Ghadiri, M. R. *Angew. Chem., Int. Ed.* **1998**, *37*, 126–128. (c) Todd, M. H. *Chem. Soc. Rev.* **2002**, *31*, 211–222.
(13) Silyl ethers are known to hydrolyze slowly in water; therefore, it remains a possibility that the background signal obtained with reagent **1** may be the consequence of a slow hydrolysis reaction followed by autocatalytic amplification of the released fluoride.
(14) (a) Kim, S. Y.; Hong, J.-I. *Org. Lett.* **2007**, *9*, 3109–3112. (b) Zhu, C.-Q.; Chen, J.-L.; Zheng, H.; Wu, Y.-Q.; Xu, J.-G. *Anal. Chim. Acta* **2005**, *539*, 311–316.
(15) Hodge, V. F.; Stallard, M. O. *Environ. Sci. Technol.* **1986**, *20*, 1058–1060.
(16) Carey, J. S.; Laffan, D.; Thomson, C.; Williams, M. T. *Org. Biomol. Chem.* **2006**, *4*, 2337–2347.
(17) (a) Duan, L.; Xu, Y.; Qian, X. *Chem. Commun.* **2008**, 6339–6341. (b) Jun, M. E.; Ahn, K. H. *Org. Lett.* **2010**, *12*, 2790–2793. (c) Li, H.; Fan, J.; Du, J.; Guo, K.; Sun, S.; Liu, X.; Peng, X. *Chem. Commun.* **2010**, *46*, 1079–1081.
(18) After 6 h, enough fluoride was present to provide an efficient amplification process in the second step. See the SI for details.