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Dendritic Chain Reaction

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The development and validation of signal amplification techniques is an important and active area of research.[1−4] These techniques are broadly used to improve the detection sensitivity of various analytes for diagnostic purposes.[5−11] While most detection methods are based on linear amplification of a measurable signal, immuno-PCR[12] (polymerase chain reaction) is a diagnostic technique that takes advantage of this process for exponential generation of DNA molecules to detect antigens with extremely high sensitivity.[13,14] Recently, Mirkin's group reported the first example of an exponential amplification assay not based on a PCR technique, which was used for the detection of acetate anion.[15] Their elegant approach employed a catalytic system that generated the analyte of interest, an acetate anion, under a carbon monoxide atmosphere in an organic solvent. Here we report a novel modular technique for exponential amplification of diagnostic signals that is conveniently performed under aqueous conditions. Our technique is based on distinctive dendritic chain reaction (DCR) that uses the disassembly properties of self-immolative dendrimers[16] (Figure 1). These dendrimers can spontaneously release their end-group molecules through domino-like reactions triggered by a single activation event.[17,18]

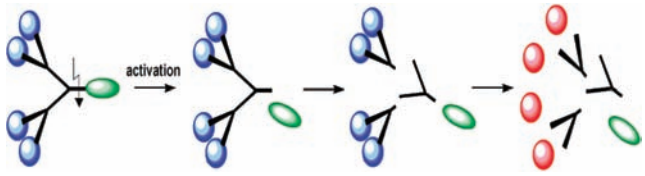


Figure 1. Schematic illustration of the activation and disassembly of self-immolative dendrimers. The trigger is shown in green and the end groups in blue. Once released, the end groups are activated (red) and able to trigger the disassembly of additional dendrimers.

Once the end groups are free, they acquire the chemical reactivity to cleave the trigger of another dendritic molecule. Therefore, a single activation event leads to a chain reaction that disassembles all of the dendritic molecules through an exponential progress, ultimately releasing all of the end groups.

The amplification principle of the DCR technique is illustrated graphically in Figure 2. The self-immolative AB_3 dendron **2** has one reporter and two reagent end groups and a trigger that can be cleaved by analyte **1**. Cleavage of the trigger by the analyte generates the release of one chromogenic reporter and two reagent molecules. The two free reagents can then activate two additional dendrons by cleavage of their triggers. These events generate two more chromogenic reporters and four additional free reagent units. The process progresses through exponential growth until all of the dendrons have been disassembled. Under ideal conditions, a single analyte molecule should be able to initiate a DCR to generate a strong diagnostic signal. On the basis of the above illustration, we synthesized the self-immolative AB_3 dendron **3**, which is designed

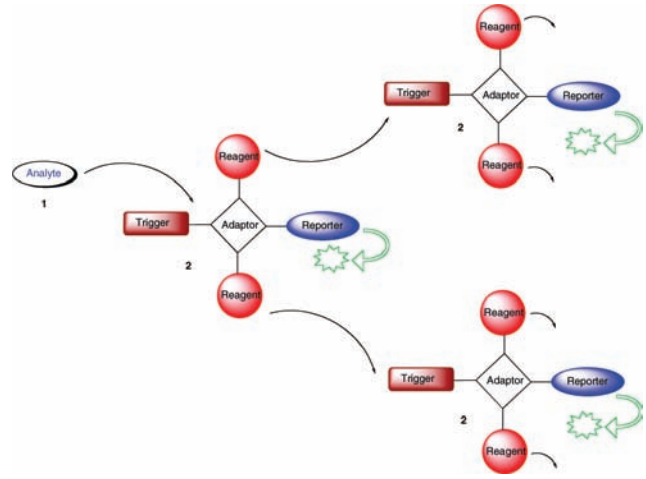
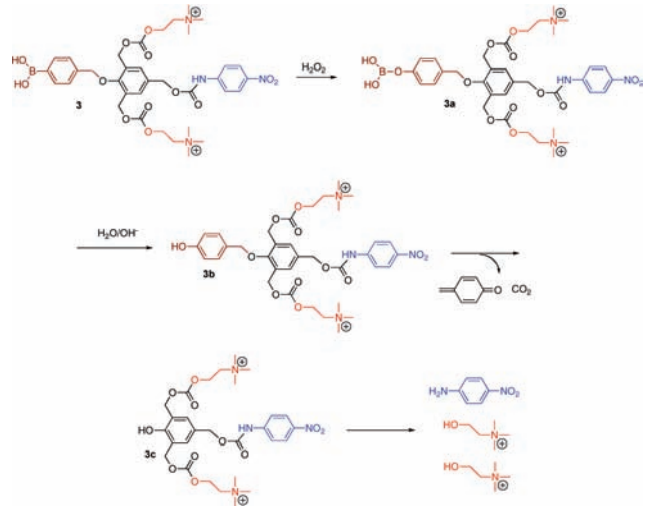


Figure 2. Graphical illustration of the DCR technique.

to detect the analyte hydrogen peroxide (Scheme 1). Dendron **3** consists of a 4-nitroaniline reporter, two choline units, and phenylboronic acid as a trigger.[19,20]

Scheme 1. Disassembly Pathway of AB_3 Self-Immolative Dendron **3**, Initiated by a Trigger That Is Cleaved by Hydrogen Peroxide (Blue, 4-Nitroaniline Reporter; Red, Choline Units That Trigger Further Dendron Disassembly)



The boronic acid moiety of **3** is known to react with hydrogen peroxide[20,21] under mild alkaline conditions to generate intermediate **3a**, which is then hydrolyzed to produce phenol **3b**. The latter undergoes 1,6-elimination and decarboxylation reactions to release phenol **3c**, which is further disassembled through triple elimination to release 4-nitroaniline and the two choline molecules.

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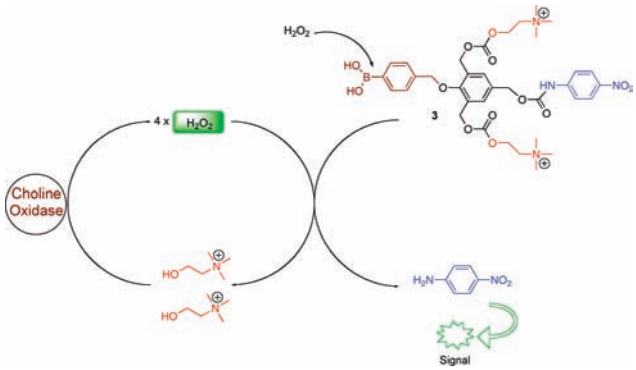

Figure 3. DCR system based on a hydrogen peroxide trigger and choline.

The DCR amplification cycle of dendron **3** is illustrated in Figure 3. Cleavage of the trigger of dendron **3** by a hydrogen peroxide molecule generates the release of one chromogenic reporter and two choline molecules. The two free choline units are oxidized by choline oxidase (COX) present in solution to produce four molecules of hydrogen peroxide, which then activate an additional four AB_3 dendrons. Betaine is also formed as the oxidized product of choline. The rate of disassembly should increase exponentially until all of the 4-nitroaniline molecules have been released. The signal can be detected by monitoring the yellow color of the released 4-nitroaniline.

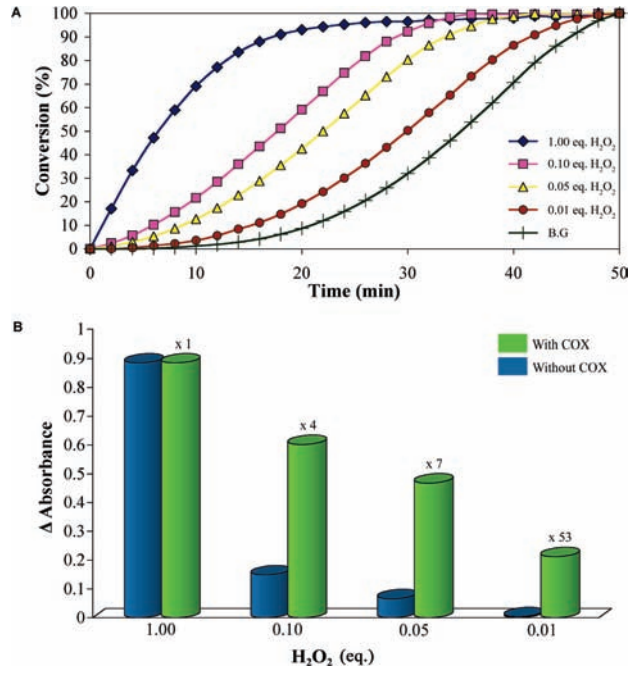

Figure 4. (A) Release of 4-nitroaniline from dendron **3** (500 μM) in the presence of COX (0.3 mg/mL) in PBS (pH 7.4) upon addition of buffer only (B.G.) or the indicated amounts of H_2O_2 monitored at 405 nm over time. (B) Comparison of signals measured in the presence of COX by the DCR amplification technique (green) vs signals measured using dendron **3** as a classic probe (i.e., without COX) (blue). The background signal present at 25 min was subtracted from the values shown.

In order to evaluate the DCR technique, dendron **3** was incubated with various amounts of hydrogen peroxide in the presence of COX, and the release of 4-nitroaniline was monitored at a wavelength of 405 nm (Figure 4A). When 1.0 equiv of hydrogen peroxide (vs dendron **3**) was used, the system reached complete disassembly within 30 min. As expected, the system reached complete disassembly after a longer period of time when less hydrogen peroxide (as little as 0.01 equiv) was used. The exponential progress of the system disassembly is clearly illustrated by the pattern of the sigmoidal plots for various amounts of H_2O_2. Since the background signal generated by spontaneous hydrolysis is also amplified, the sensitivity of this system allowed detection of analyte down to only 5 μM H_2O_2. The increased sensitivity of the DCR technique is clearly demonstrated when the background noise is subtracted from the measured signal and presented next to a signal obtained without the addition of COX (Figure 4B). When 1.0 equiv of the analyte was applied with no added COX, dendron **3** exhibited the behavior of a classic probe (a molecule constructed from a triggering protecting group attached to a reporter) and generated a signal level identical to that measured using the DCR. However, when decreasing amounts of hydrogen peroxide were applied, the signal measured in the presence of COX by the DCR technique was significantly larger than that obtained from the classic probe. In fact, the ratio of the DCR signal to the classic one increased with decreasing analyte concentration (limited by the system sensitivity). In the limit of the assay sensitivity, with 0.01 equiv of hydrogen peroxide, the signal of the DCR technique was 53-fold stronger than that obtained by the classic probe. In this representative assay (in PBS at pH 7.4 with a reaction volume of 100 μL and probe concentration of 500 μM), upon reaction with a single molecule of hydrogen peroxide, 28 cycles were required for completion of the disassembly of all of the DCR probe molecules (as calculated by the sum equation for a geometric series). This dendritic chain reaction can be viewed as the disassembly of a virtual dendrimer of 2^{n-1} generation, where n is the number of cycles needed to complete the disassembly of all of the dendritic molecules present in the solution.

The DCR-based assay presented here can be extended to the detection of other analytes and biocatalysts simply by coupling it with another probe activity. For example, release of a choline molecule by a specific protease in the presence of COX and DCR probe **3** can serve as the basis for an assay to detect that protease activity. Accordingly, DCR probe **3** could be used as a core system for constructing diagnostic assays for other analytes. To demonstrate this possibility, we synthesized probe **4** (shown at the top of Figure 5), in which choline is attached to phenylacetamide, a substrate of the enzyme penicillin-G-amidase (PGA). Cleavage of the phenylacetamide by PGA followed by spontaneous 1,6-elimination and decarboxylation releases a choline molecule. Various concentrations of PGA were incubated with probe **4** together with DCR probe **3** and COX. The release of 4-nitroaniline was monitored at a wavelength of 405 nm (Figure 5A). The obtained sigmoidal plots confirmed the coupling of the proteolytic assay with the DCR amplification technique. PGA was detected at concentrations as low as 10^{-4} mg/mL. Figure 5B shows the relative difference in signals obtained by the DCR technique and a classic approach (using a probe in which phenylacetamide is directly attached to 4-nitroaniline).

The principles of the DCR system are based on autoamplification generated by release of small molecules. Once these molecules are free, they acquire the ability to trigger the disassembly of additional probes and thereby initiate new diagnostic cycles. The DCR approach offers considerable advantages in comparison with diagnostic probes based on a stoichiometric reaction between the analyte and the probe. For example, the reaction demonstrated in this study, between the analyte, hydrogen peroxide, and a phenylboronic acid probe, occurs stoichiometrically. The detection of such analytes can be significantly improved by applying the DCR

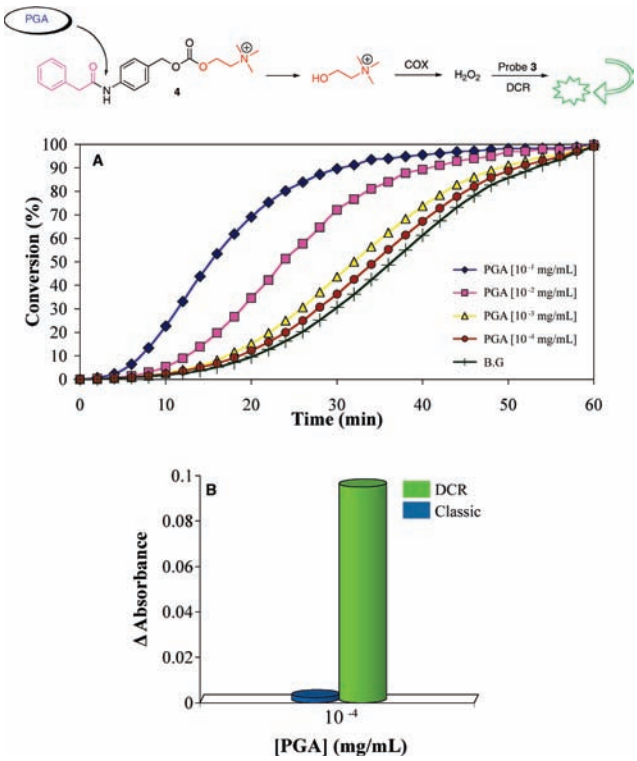

Figure 5. Diagnostic assay for detection of PGA through coupling with a DCR probe. (A) Release of 4-nitroaniline from dendron **3** (500 μM) in the presence of COX (0.3 mg/mL) and probe **4** (500 μM) in PBS at pH 7.4 monitored at 405 nm over a range of PGA concentrations. (B) Comparison of the signal measured in the presence of COX by the DCR amplification technique (green) vs that measured using phenylacetamide directly attached to 4-nitroaniline (blue) at 30 min. The values shown were obtained by subtracting the background at 30 min.

technique, since the stoichiometric reaction of the analyte and the probe can be exponentially amplified to generate a strong diagnostic signal.

In most diagnostic assays, the detection limit of the analyte is dependent on the signal-to-noise ratio. The sensitivity of the DCR assay is also limited by the spontaneous hydrolysis of the probe, which is then amplified. Most likely, the background signal is generated through hydrolysis of the carbonate linkage of the choline in dendron **3** (in a control experiment, hydrogen peroxide did not show any carbonate cleavage under the DCR assay conditions). However, in the substrate concentration range presented in Figures 4 and 5, the detection sensitivity was still higher for the DCR probe than for the classic probe. Going to lower concentrations of analyte would indeed result in better detection sensitivity with a classic probe such as phenacetamide or nitroaniline. Although there are systems for the detection of hydrogen peroxide (e.g., Amplex Red or horseradish peroxidase assays) that are more sensitive than the current DCR probe, further development and optimization can significantly improve the detection sensitivity of this technique. The modularity of the probe molecule should allow the replacement of structural elements; for example, the choline substrate could be replaced with other compounds that can be oxidized to produce hydrogen peroxide, thereby allowing the application of other oxidases in the DCR assay. In principle, any analyte of interest that has cleavage reactivity toward a specific trigger could be incorporated collectively with the specific triggering group in the dendritic platform.

In conclusion, we have demonstrated a novel technique for signal amplification that, like immuno-PCR, takes advantage of exponential growth. The amplification is based on a distinctive dendritic chain reaction that progresses through a growing number of diagnostic cycles. This is the first example of exponential signal amplification for diagnostic purposes that is not based on PCR and can be performed under aqueous conditions. When coupled with an additional probe activity, this technique could be applied for detection of other analytes. Optimization of the aqueous stability of the probe with respect to spontaneous hydrolysis while maintaining a rapid disassembly rate upon reaction with the analyte should significantly increase the sensitivity of the DCR technique.

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Supporting Information Available: Full experimental details and characterization data for all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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