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Enzymatic activation of hydrophobic self-immolative dendrimers: The effect of reporters with ionizable functional groups

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ABSTRACT

Self-immolative dendrimers are uniquely structured molecules that release multiple tail units through a chain fragmentation initiated by a single cleavage at the dendrimer's core. Although bioactivation of self-immolative dendritic molecules with only two reporter groups was demonstrated, enzymatic activation failed for self-immolative dendrimers with more reporters. These large and hydrophobic dendrimers aggregated under aqueous conditions and enzyme did not efficiently trigger chain fragmentation. Here we demonstrate a simple solution to the problem of enzymatic activation of hydrophobic self-immolative dendrimers. The reporter units on the dendritic platform were equipped with ionizable functional group. Polar interactions with water significantly decreased hydrophobicity of the dendrimers and prevented aggregate formation. Consequently, hydrophobic self-immolative dendrons were effectively activated.

Self-immolative dendrimers are designed to release multiple peripheral end-units upon a single activation event at the dendrimer's focal point. This concept was first explored six years ago, almost simultaneously, by three different research groups including ours.[1–3] All three groups exploited the fact that the dendrimer skeleton can be constructed in such a way that the entire molecule will disintegrate into known fragments once the disintegration process is initiated. Incorporation of drug molecules as the tail units and use of an enzyme substrate as the trigger generates a multi-prodrug that is activated with a single enzymatic cleavage. These dendritic prodrugs offer significant advantages in comparison with classic monomeric prodrugs in inhibition of tumor cell growth.[4,5] We have also designed and synthesized fully biodegradable dendrimers that disassemble through multi-enzymatic triggering followed by self-immolative chain fragmentation.[6,7] A practical application for such multi-triggered self-immolative dendrons was demonstrated by the concept of prodrug activation through a molecular OR logic trigger.[8] Self-immolative dendrimers were also shown to act as efficient molecular amplifiers in the design of diagnostic probes.[9,10] Recently, we harnessed these dendrimers as a platform for controlled self-assembly of peptide nanotubes.[11]

The building blocks of self-immolative dendrimers are usually hydrophobic aromatic molecules and, therefore, these molecules have poor water solubility and tend to aggregate under aqueous conditions.[12] When aggregation occurs, access of the enzyme to the substrate at the dendron's focal point is restricted and fragmentation is inefficient. We recently demonstrated that enzymatic activation of second-generation self-immolative dendrimers can be achieved by conjugation of polyethylene glycol to the dendritic platform.[13] The polyethylene glycol tails significantly decrease the hydrophobic properties of the dendrimers and prevent aggregate formation. Here we report second approach to achieve enzymatic activation of these dendrimers. We hypothesized that an ionizable functional group introduced into the reporter unit would significantly increase aqueous solubility and prevent aggregate formation. In order to evaluate our approach, we designed self-immolative dendritic molecules **1**, **2**, and **3** (Fig. 1). The dendrons were constructed with an enzymatic trigger that is activated by Penicillin-G-Amidase(PGA)[14] and the reporter groups were 5-amino-2-nitrobenzoic acid.

The dendrons were designed to undergo complete disassembly to release their reporter units upon a single cleavage event by PGA. The disassembly mechanism of dendron **3** is presented in Scheme 1. Following the enzymatic cleavage, aza-quinonemethide is rapidly eliminated and decarboxylation occurs, leading to release of amine **3a**. The latter is disassembled through double 1,6 and 1,4-eliminations to generate two equivalents of amine **3b**, which is further fragmented to release four equivalents of 5-amino-2-nitrobenzoic acid.

Due to aggregate formation, analog dendrons equipped with 4-nitroaniline reporter units instead of 5-amino-2-nitrobenzoic acid were activated by PGA only for zeroth-generation molecules. The addition of an ionized carboxylic acid to the reporters is expected to inhibit aggregate formation otherwise observed in aqueous conditions and thereby to allow activation of first and second-generation dendrons by the enzyme.

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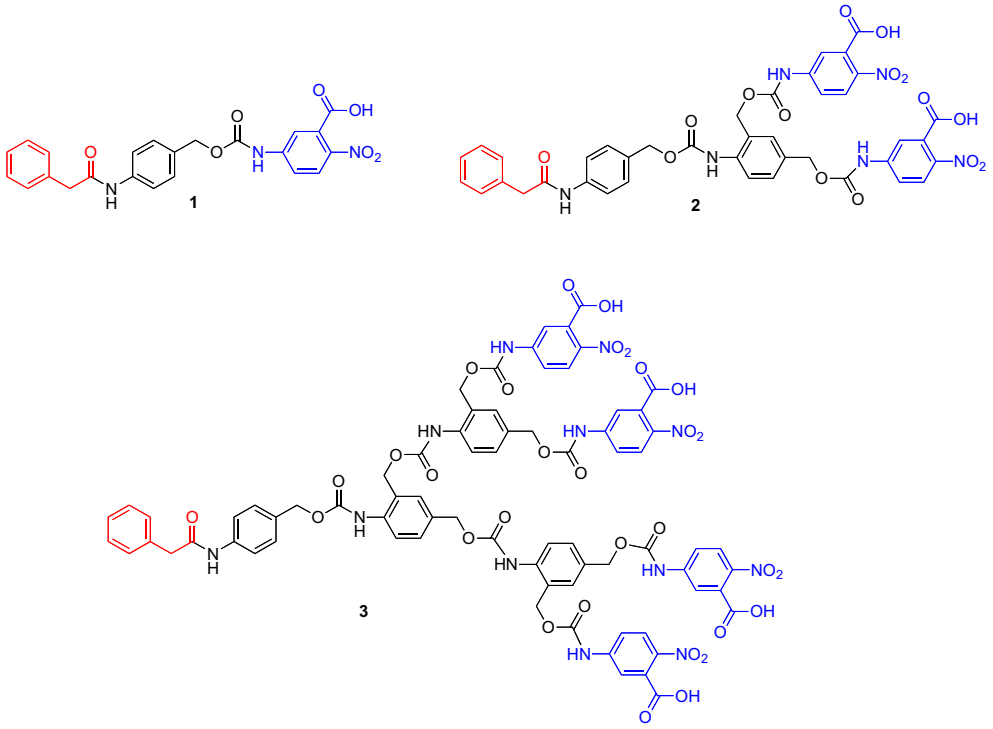
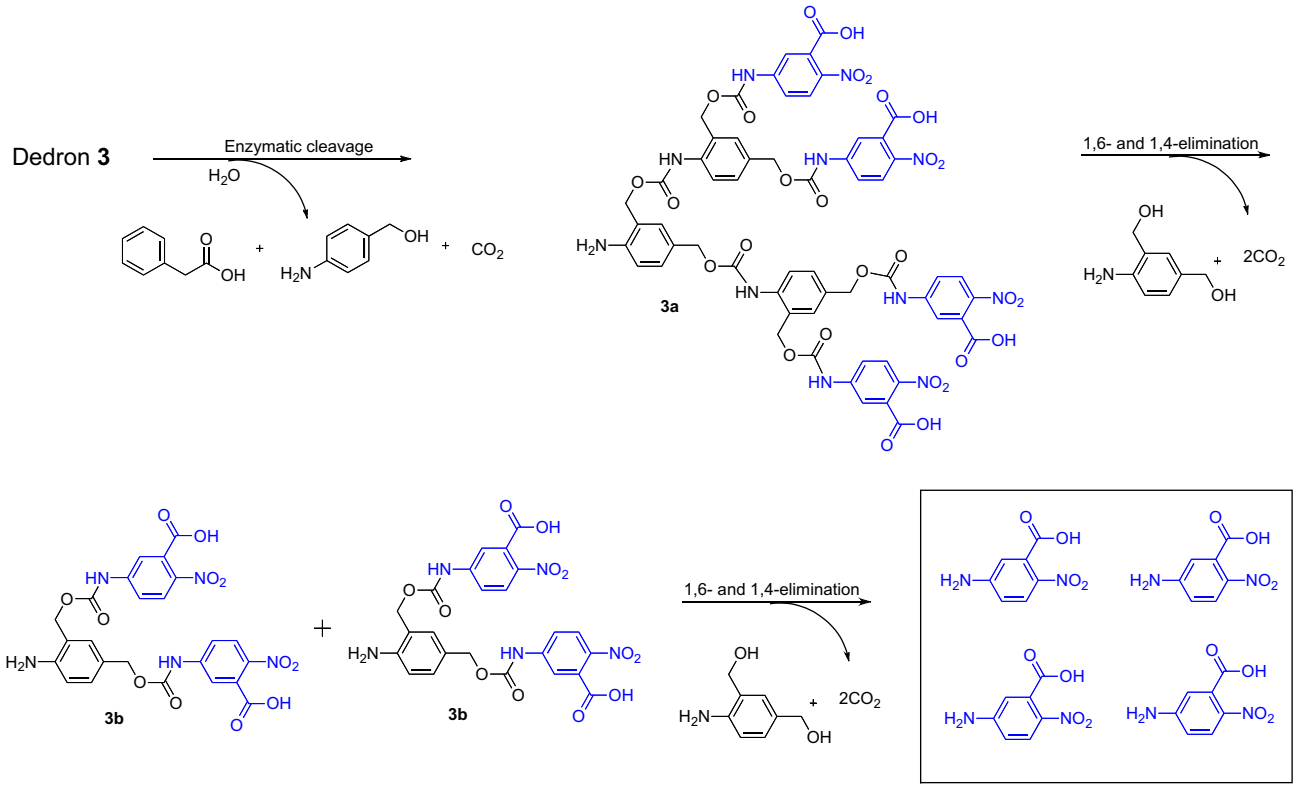
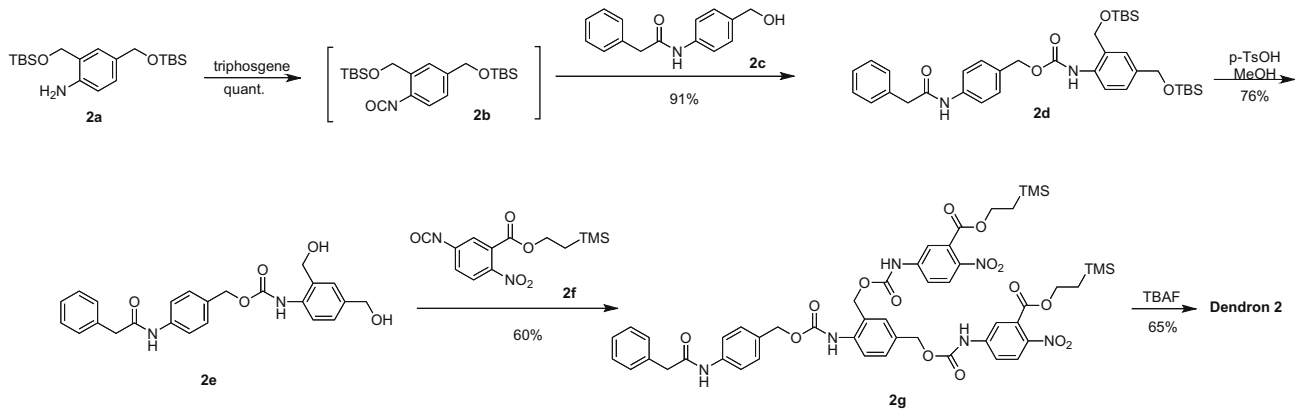


Figure 1. Chemical structures of zeroth-generation (**1**), first-generation (**2**) and second-generation (**3**) self-immolative dendrons. The substrate for PGA is shown in red and the 5-amino-2-nitrobenzoic acid reporter groups are shown in blue.



Scheme 1. Disassembly pathway of second-generation dendritic molecule **3** triggered by enzymatic activation of PGA.

The synthesis of compound **1** was performed in a manner similar to the previously published procedure (see Supplementary data).[15] Dendron **2** was synthesized as shown in Scheme 2. Amine **2a**[15] was treated with triphosgene to generate isocyanate **2b**, which was coupled in situ with alcohol **2c** to afford carbamate **2d**. Deprotection of carbamate **2d** in the presence of *p*-



Scheme 2. Chemical synthesis of dendron **2**.

toluenesulfonic acid generated diol **2e**. Then compound **2f** (prepared from the corresponded amine) was coupled in situ with alcohol diol **2e** to afford compound **2g**. The latter was deprotected with tetrabutylammonium-fluoride to give first-generation dendron **2**.

The synthesis of second-generation dendron **3** is presented in Scheme 3. Two equivalents of isocyanate **2b** were coupled with diol **2e** to give dendron **3c**. Deprotection of compound **3c** in the presence of *p*-toluenesulfonic acid generated tetra-ol **3d**. An excess of isocyanate **2f** was coupled with tetra-ol **3d** to afford compound **3e**. The latter was deprotected by tetrabutylammonium-fluoride to yield second-generation dendron **3**.

Since the free reporter 5-amino-2-nitrobenzoic has an absorbance at wavelength of 405 nm, the disassembly of self-immolative dendrons **1**–**3** can be monitored by Vis-spectroscopy. Dendrons **2** and **3** were incubated in phosphate buffered saline (PBS, pH 7.4) in the presence of PGA and disassembly was monitored. The results are presented in Figure 2. Dendron **2** rapidly disassembled; all reporter units were released within 30 min. Disassembly of dendron **3** occurred over a longer time but, as expected, released double the number of reporter units in comparison to dendron **2**. No release of 5-amino-2-nitrobenzoic was observed when either dendron was incubated in absence of PGA (data not shown).

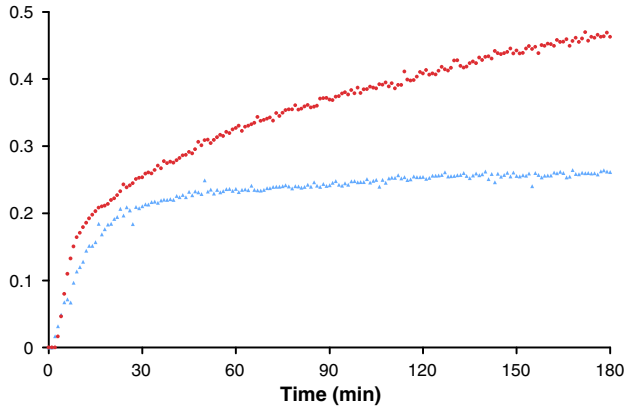
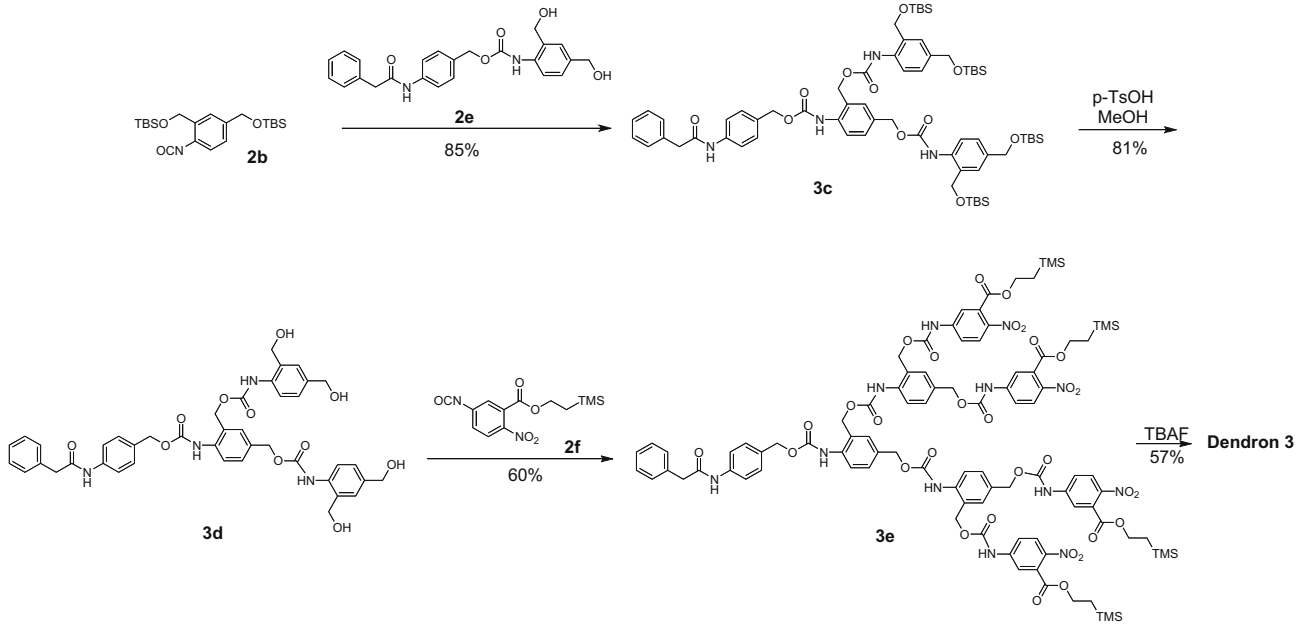


Figure 2. PGA-catalyzed the release of 5-amino-2-nitrobenzoic acid from dendrons **2** (▲) and **3** (●). Conditions: 250 μM **2** or **3**, 0.01 mg mL^{-1} PGA in PBS (pH 7.4). Absorbance was monitored at 405 nm.



Scheme 3. Chemical synthesis of dendron **3**.

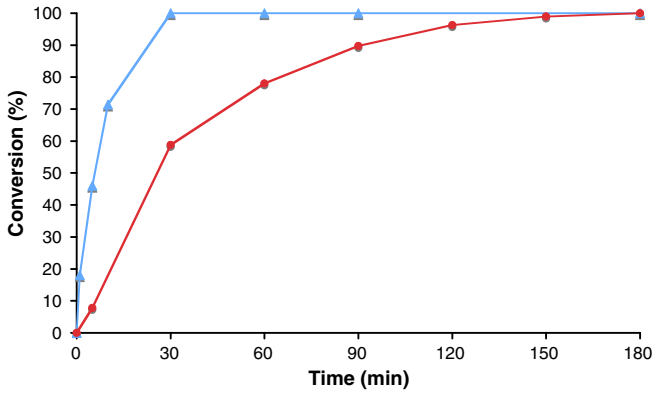


Figure 3. HPLC analysis PGA-catalyzed the release of 5-amino-2-nitrobenzoic acid from compound **2** (▲) and **3** (●). Conditions: 250 μM **2** or **3**, 0.01 mg mL^{-1} PGA in PBS (pH 7.4). Absorbance was monitored at wavelength of 348 nm. Separation was performed on a C-18 RP-column (gradient: 30–100% ACN in water over 20 min).

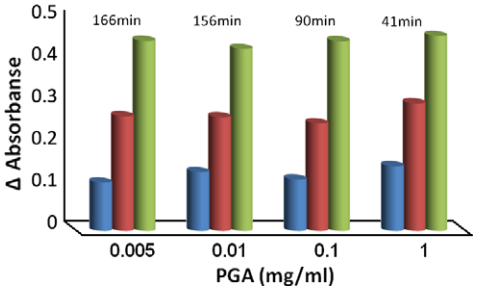


Figure 4. Change in absorbance (at 405 nm) as a function of PGA concentration for the catalyzed release of 5-amino-2-nitrobenzoic acid from dendron **1** (●), dendron **2** (●), and dendron **3** (●). The data shown are for the time point after disassembly was complete at each concentration of enzyme. Conditions: 250 μM dendron **1**, **2**, or **3** in PBS (pH 7.4) with indicated concentration of PGA.

To confirm the observed spectroscopic results, we also monitored the disassembly of the dendrons in the presence of PGA by reversed-phase HPLC assay (Fig. 3). This analysis also indicated that dendron **2** was completely disassembled more rapidly than dendron **3**. The reaction of **2** was completed within 30 min, whereas dendron **3** was completely disassembled after 3 h.

Finally, we evaluated the disassembly of zeroth-generation dendron **1**, first-generation dendron **2**, and second-generation dendron **3** using different concentrations of PGA. The results are presented in Figure 4. At higher concentrations of PGA, reactions were faster. As expected, second-generation dendron **3** and first-generation dendron **2** generated fourfold and twofold stronger spectroscopic signals, respectively, than did zeroth-generation dendron **1**.

Like the effect obtained by PEGylation of self-immolative dendrimers,[13] addition of ionizable functional groups to the reporter units presumably inhibits aggregation and enhances solubility of these hydrophobic molecules in water. We demonstrate that use of this approach allowed enzymatic activation of hydrophobic self-immolative dendrimers with four reporter units per dendron. This type of dendritic molecule should be useful as a molecular probe for detection of specific activity by introduction of the appropriate trigger in the dendrimer focal point. The reporter unit release can be conveniently monitored by UV-spectroscopy.[16–20] The sensitivity of the molecular probe is enhanced at higher dendrimer generations.

In summary, we have demonstrated a new approach to achieve enzymatic activation of second-generation self-immolative dendrimers based on use of reporter units with ionizable functional groups. We designed and synthesized zeroth-, first-, and second-generation dendritic molecules with 5-amino-2-nitrobenzoic acid as a reporter and a trigger that allows activation by PGA. All three dendritic molecules, including the second-generation self-immolative dendron was otherwise inactive due to aggregation, were effectively cleaved by PGA in aqueous solution. The data presented in this study demonstrate that self-immolative dendrimers, with reporters monitored by UV, can be used as molecular probes for detection of various enzymatic activities.

Acknowledgments

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2009.03.002.

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