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Activatable Dendritic ^{19}F Probes for Enzyme Detection

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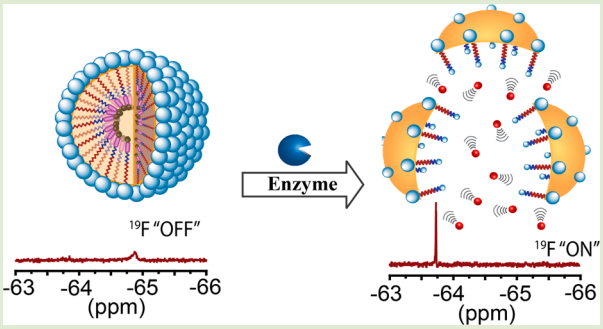
Supporting Information

ABSTRACT: We describe a novel activatable probe for fluorine-19 NMR based on self-assembling amphiphilic dendrons. The dendron probe has been designed to be spectroscopically silent due to the formation of large aggregates. Upon exposure to the specific target enzyme, the aggregates disassemble to give rise to a sharp ^{19}F NMR signal. The probe is capable of detecting enzyme concentrations in the low nanomolar range. Response time of the probe was found to be affected by the hydrophilic−lipophilic balance of dendrons. Understanding the structural factors that underlie this design principle provides the pathway for using this strategy for a broad range of enzyme-based imaging.



Detection and imaging of enzyme activity is of great importance in drug discovery research and medical diagnostics.[1] Activatable probes, which induce a signal change in response to a specific stimulus, are promising for imaging enzymatic activity because they provide high sensitivity and selectivity.[2] Most of the activatable probes developed so far are based on fluorescence.[3] However, low penetration depth, caused by scattering, imposes some limitations for fluorescence-based *in vivo* imaging. On the other hand, activatable probes based on NMR spectroscopy are gaining interest, as this is the first indicator that a probe could be viable for magnetic resonance imaging (MRI) based deep-tissue visualization.[4] There have been efforts to develop activatable probes for enzymes using gadolinium-based reagents, where the enzyme activity changes the coordination sphere of Gd(III).[4b,5] This causes changes in the ^{1}H relaxation times of the bound water molecules providing a magnetic resonance signal. Although several Gd-based reagents are in the clinic, recent concerns over these as causative agents for nephrogenic systemic fibrosis[6] have triggered the search for alternate imaging modalities. Fluorine-19 has emerged as a promising NMR-active nucleus for this purpose[7] because of its high isotopic abundance (100%) and also because fluorine is not naturally found in detectible amounts in physiological fluids. The negligible presence of endogenous ^{19}F endows this strategy with the possibility of high contrast, a critical feature for imaging. Despite its great potential, ^{19}F NMR strategies for specifically detecting enzymatic activities are very limited.[8] In this communication, we introduce a novel ^{19}F NMR strategy for detecting enzyme activity based on self-assembled facially amphiphilic dendrons and identify the key structural features that control enzyme-induced signal generation.

Our approach is schematically shown in Figure 1, which is based on self-assembling facially amphiphilic dendrons.[9] In this study, these dendrons are designed such that the fluorine-bearing hydrophobic functionalities are buried within the interior of the amphiphilic aggregate. However, the ability of these dendrons to aggregate would be compromised, when an enzymatic reaction alters the hydrophilic−lipophilic balance of the dendron. We hypothesize that this transformation will provide a significant change in the magnetic resonance behavior of the fluorine nuclei, since the transverse (T_2) relaxation in NMR is very sensitive to molecular weight.[10] The formation of a large molecular assembly from the facially amphiphilic dendron would cause severe broadening and attenuation of the ^{19}F signal as shown in Figure 1. However, upon specific enzymatic reaction the dendrons would release a small molecule that gives rise to a sharp and intense signal.

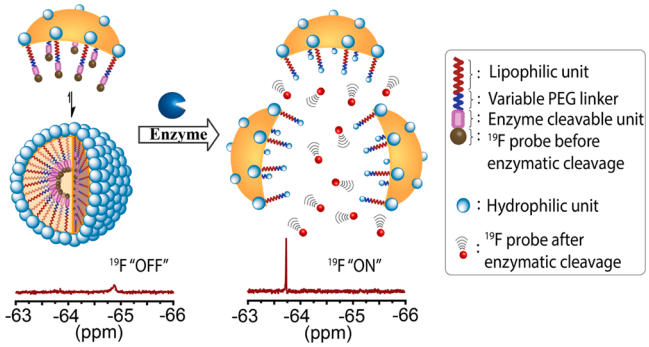


Figure 1. Schematic representation of assemblies formed from enzyme-cleavable dendrons and the release of the ^{19}F reporter upon enzyme exposure.

Binding-induced deaggregation strategies have been previously attempted for sensing and imaging.[7e,9d] However, reactivity-based strategies targeted here provide an additional challenge. The key functional groups that are responsible for

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signal generation, i.e., the substrate functionalities for the enzyme, are buried in the interior of a large aggregate.[9c,11] It is critical that enzymes have access to these substrates, yet it is also important that the molecules exist mostly in the aggregate form in order to provide a fully turned-off signal, prior to encountering the enzyme. We chose dendritic scaffolds because they provide a unique opportunity to address this challenge.[12] Dendrimers have the advantage of providing low critical aggregation concentrations, similar to those observed with polymers, but provide the molecular weight control that is often possible only with small molecules.[13] In addition, dendrimers also afford a high degree of control over functional group placements within a macromolecule.[14] These features allow for understanding the fundamental structural dependencies in sensitive signal generation, induced by specific enzymatic triggers.

To test our design hypothesis, we synthesized dendron **1** containing three 3,5-bis(trifluoromethyl)phenyl moieties as a hydrophobic side chain in the dendron (Figure 2). Each of

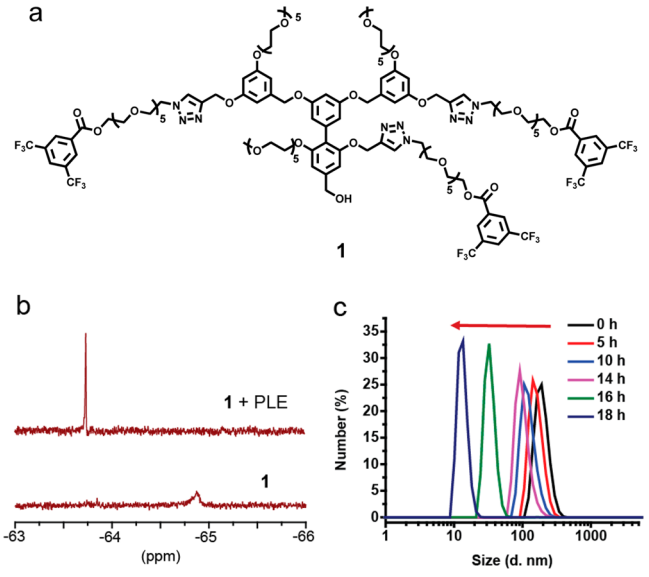


Figure 2. (a) Chemical structure of enzyme-cleavable dendron 1. (b) ^{19}F NMR spectra of **1** (25 μM) in the presence or absence of PLE (1 μM) (TFA as an internal standard for chemical shift). (c) Size evolution of **1** (25 μM) in the presence of PLE (1 μM) using DLS.

these functional groups carries six magnetically equivalent ^{19}F nuclei. The pentaethylene glycol unit was used as the hydrophilic unit, as these charge-neutral functionalities do not exhibit nonspecific interactions with proteins and enzymes. An ester functionality was installed as the enzyme-cleavable substrate. In the absence of target enzyme, dendron **1** self-assembles into a large aggregate, which results in a very weak and broad ^{19}F signal. Upon exposure to porcine liver esterase (PLE), the enzymatic reaction should cleave the ester bond to liberate 3,5-bis(trifluoromethyl)benzoic acid. Indeed, when **1** (25 μM) was dissolved in a buffer solution, a weak and broad ^{19}F NMR signal was observed initially (Figure 2b). However, a sharp signal appeared at −63.7 ppm upon addition of PLE (1 μM). This sharp and strong ^{19}F signal, obtained in the reaction mixture, is indicative of the formation of the small molecule. To further validate the potential of this specific enzyme-responsive signal enhancement in the context of its ultimate use in MRI applications, we attempted to measure the t_2 relaxation time of the sharp signal and found a value of 1.3 s, which is typical for small molecules. This underlines the extent of signal generation in the presence of the enzyme and the very low background signal in the absence of the enzyme. Furthermore, we also determined that the ^{19}F signal intensity is proportional to PLE concentration and that the detection limit is 1 nM PLE (Figure 3b). In addition to the generation of the fluorine-containing

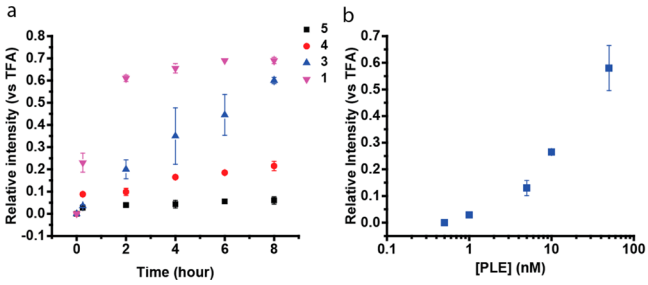
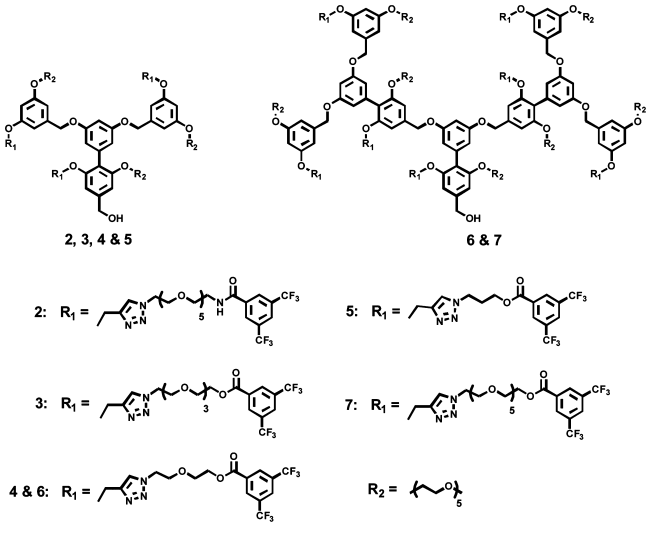


Figure 3. (a) Temporal evolution of ^{19}F NMR intensity (−63.7 ppm) of G1 dendrons (25 μM) treated with PLE (1 μM) over the first 8 h. (b) Dependence of the ^{19}F NMR intensity (−63.7 ppm) on PLE concentration for dendron **1** (25 μM) (measurements taken after 24 h PLE incubation). All experiments were performed in 25 mM Tris buffer (pH 7.4, 0.2 mM TFA as an internal standard for peak intensity and chemical shift, 10% D_2O (v/v)) at 25 °C.

small molecule, the enzymatic reaction also cleaves the most hydrophobic part of the amphiphilic dendron. This modification changes the hydrophilic−lipophilic balance of the dendron and converts it to a much more hydrophilic one. This should cause the size of the assembly to change significantly. We used dynamic light scattering to measure the size change of the dendron before and after enzyme incubation. As shown in Figure 2c, the assembly size of **1** (25 μM) alone was ∼200 nm (Figure S1, Supporting Information), whereas the size decreases systematically over time upon addition of PLE (1 μM), validating the enzyme-induced disassembly of the aggregates.

To further confirm that ^{19}F signal and the disassembly were indeed caused by the enzymatic hydrolysis of ester functionalities, we synthesized a structurally similar dendron (Chart 1), **2**, in which ester moieties were replaced by amides. Since PLE

Chart 1. Structures of ^{19}F-Containing Amphiphilic Dendrons



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should not be able to cleave an amide bond, we anticipated that neither the ^{19}F signal nor the assembly size of dendron **2** would be affected by PLE. Indeed, no new ^{19}F signal appeared, and the assembly size stayed the same after dendron **2** (25 μM) was incubated with PLE (1 μM) (Figure S2, Supporting Information). Similarly, to determine whether the signal generation is specific to PLE, we exposed dendron **1** (25 μM) to four additional proteins (1 μM) with varying pI values, viz., myoglobin (pI 7.2), hemoglobin (pI 6.8), avidin (pI 10.5), and pepsin (pI 1.0). As expected, none of these proteins showed any ^{19}F signal generation (Figure S5, Supporting Information), underlining the specificity of this dendron probe to only the target enzyme.

If the enzyme-induced deaggregation was correct, then it is critical that the enzyme has access to the ester moiety that is buried in the hydrophobic interior of the aggregate. We hypothesize that it is the unimer−aggregate equilibrium that provides the pathway for the enzymatic access to the substrate moiety and the ensuing deaggregation. To test this hypothesis and thus provide the molecular design guidelines for optimal sensitivity, we evaluated the effect of modulating the hydrophilic−lipophilic balance of the dendron upon the response time in signal generation. Dendrons **3**−**7** were synthesized to test these possibilities (Chart 1). First of all, note that dendron **1** contains an oligoethylene glycol moiety on both faces of the dendron with the presence of the bis(trifluoromethyl)phenyl moiety as the key difference between the more hydrophilic and hydrophobic faces of the dendron. When we utilized the more classical hydrophobic linker, as shown in **5**, no signal was generated even after 8 h. A signal was observed after incubation of the enzyme for 4 days (Figure S4, Supporting Information), which suggests that the esterase-induced signal generation is indeed possible but is very slow. This observation is likely due to the increased hydrophobicity of the dendron, which makes the unimer state of the dendron less available. This is consistent with our mechanistic hypothesis.

To further test this, we studied dendrons **3** and **4**, where the length of the oligoethylene glycol unit side chain was systematically changed compared to that in **1**. These dendrons were incubated with PLE, and the temporal evolution of the ^{19}F NMR signal was monitored. As expected, the dendron **1** has the fastest signal evolution, where the signal was saturated in just 6 h. Signal evolution from dendron **3** was slower than that from **1** but faster than that from **4** (Figure 3a). The size evolution of each of these dendrons was also monitored by DLS (Figure S5, Supporting Information). Upon incubation with PLE, the assembly size of **3** reduced from ∼200 to ∼30 nm after 17 h. The size reduction of **4** was slower compared to that of **3** and **1**; it took ∼26 h to reach 30 nm. As anticipated, the aggregate size of dendron **5** reduced only slightly even after 96 h. All these results are consistent with our mechanistic hypothesis that the signal generation relies on the hydrophilic lipophilic balance of the dendron, which likely affects the unimer−aggregate equilibrium-based activation.

Finally, we investigated generation dependence upon the probe response time. G2 dendrons are potentially more sensitive than G1 dendrons as the number of ^{19}F nuclides per dendron unit is more than twice the amount in G1 dendrons. However, similar to increased hydrophobicity causing the equilibrium concentration of the unimer to be smaller, we also anticipated that the unimer equilibrium concentration in G2 dendrons is smaller than that of G1 dendrons. This expectation is based on previous observations that higher generation dendrons exhibit longer residence time in an aggregate, compared to lower generation dendrons.[15] Accordingly, we tested G2 dendrons **6** and **7**. No signal generation was observed for these dendrons, even after PLE incubation for 4 days (Figure S4, Supporting Information).

In summary, we have shown that (*i*) by incorporating fluorine-containing hydrophobic units within amphiphilic aggregates of facially amphiphilic dendrons the ^{19}F NMR signal can be made weak and broad; (*ii*) enzyme-induced cleavage of the fluorinated moiety results in the spontaneous generation of a strong and sharp signal; (*iii*) the signal generation is specific to the enzyme for which the linker is engineered; (*iv*) the equilibrium concentration of the unimer in the unimer−aggregate equilibrium plays a key role in the kinetics of signal generation; and (*v*) the dendron probe is capable of detecting enzyme concentrations in the low nanomolar range. The activatable probe described here and the structural factors that control the signal generation are sufficiently general that this method can be conveniently elaborated to other enzymes. With the increasing potential for the development of ^{19}F MRI for clinical applications, activity-based imaging would play an important future role. Our findings here constitute a promising step for such imaging applications. In combination with the fact that these dendrons are capable of sequestering other guest molecules, these molecules can also be expanded to theranostic applications.

■ ASSOCIATED CONTENT

Supporting Information

Synthetic procedures and characterizations of the nanoparticle. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) (a) Vitzthum, F.; Behrens, F.; Anderson, N. L.; Shaw, J. H. *J. Proteome Res.* **2005**, *4*, 1086. (b) Roy, R.; Yang, J.; Moses, M. A. *J. Clin. Oncol.* **2009**, *27*, 5287. (c) Lee, D.-H.; Blomhoff, R.; Jacobs, D. R. *Free Radical Res.* **2004**, *38*, 535.

(2) (a) Kobayashi, H.; Ogawa, M.; Alford, R.; Choyke, P. L.; Urano, Y. *Chem. Rev.* **2010**, *110*, 2620. (b) Lavis, L. D.; Raines, R. T. *ACS Chem. Biol.* **2008**, *3*, 142.

(3) (a) Weissleder, R.; Tung, C. H.; Mahmood, U.; Bogdanov, A. *Nat. Biotechnol.* **1999**, *17*, 375. (b) Chen, J.; Tung, C.-H.; Allport, J. R.; Chen, S.; Weissleder, R.; Huang, P. L. *Circulation* **2005**, *111*, 1800. (c) Hu, H.-Y.; Gehrig, S.; Reither, G.; Subramanian, D.; Mall, M. A.; Plettenburg, O.; Schultz, C. *Biotechnol. J.* **2014**, *9*, 266. (d) Gao, W.; Xing, B.; Tsien, R. Y.; Rao, J. *J. Am. Chem. Soc.* **2003**, *125*, 11146. (e) Kamiya, M.; Kobayashi, H.; Hama, Y.; Koyama, Y.; Bernardo, M.; Nagano, T.; Choyke, P. L.; Urano, Y. *J. Am. Chem. Soc.* **2007**, *129*, 3918. (f) Silvers, W. C.; Prasai, B.; Burk, D. H.; Brown, M. L.; McCarley, R. L. *J. Am. Chem. Soc.* **2013**, *135*, 309.

(4) (a) Perez, J. M.; Josephson, L.; O'Loughlin, T.; Högemann, D.; Weissleder, R. *Nat. Biotechnol.* **2002**, *20*, 816. (b) Louie, A. Y.; Huber, M. M.; Ahrens, E. T.; Rothbacher, U.; Moats, R.; Jacobs, R. E.; Fraser,

DOI: 10.1021/acsmacrolett.5b00199
ACS Macro Lett. 2015, 4, 422−425

S. E.; Meade, T. J. *Nat. Biotechnol.* **2000**, *18*, 321. (c) Yang, C.-T.; Chuang, K.-H. *Med. Chem. Commun.* **2012**, *3*, 552.
(5) (a) Chang, Y. T.; Cheng, C. M.; Su, Y. Z.; Lee, W. T.; Hsu, J. S.; Liu, G. C.; Cheng, T. L.; Wang, Y. M. *Bioconjugate Chem.* **2007**, *18*, 1716. (b) Giardiello, M.; Lowe, M. P.; Botta, M. *Chem. Commun.* **2007**, 4044. (c) Yoo, B.; Pagel, M. D. *J. Am. Chem. Soc.* **2006**, *128*, 14032.
(6) (a) Grobner, T. *Nephrol., Dial., Transplant* **2006**, *21*, 1104. (b) Grobner, T.; Prischl, F. C. *Kidney Int.* **2007**, *72*, 260.
(7) (a) Yu, J.-X.; Hallac, R. R.; Chiguru, S.; Mason, R. P. *Prog. Nucl. Magn. Reson. Spectrosc.* **2013**, *70*, 25. (b) Ahrens, E. T.; Flores, R.; Xu, H.; Morel, P. A. *Nat. Biotechnol.* **2005**, *23*, 983. (c) Thurecht, K. J.; Blakey, I.; Peng, H.; Squires, O.; Hsu, S.; Alexander, C.; Whittaker, A. K. *J. Am. Chem. Soc.* **2010**, *132*, 5336. (d) Higuchi, M.; Iwata, N.; Matsuba, Y.; Sato, K.; Sasamoto, K.; Saido, T. C. *Nat. Neurosci.* **2005**, *8*, 527. (e) Takaoka, Y.; Sakamoto, T.; Tsukiji, S.; Narazaki, M.; Matsuda, T.; Tochio, H.; Shirakawa, M.; Hamachi, I. *Nat. Chem.* **2009**, *1*, 557.
(8) (a) Tanabe, K.; Harada, H.; Narazaki, M.; Tanaka, K.; Inafuku, K.; Komatsu, H.; Ito, T.; Yamada, H.; Chujo, Y.; Matsuda, T.; Hiraoka, M.; Nishimoto, S. *J. Am. Chem. Soc.* **2009**, *131*, 15982. (b) Tanabe, K.; Harada, H.; Narazaki, M.; Tanaka, K.; Inafuku, K.; Komatsu, H.; Ito, T.; Yamada, H.; Chujo, Y.; Matsuda, T.; Hiraoka, M.; Nishimoto, S. *J. Am. Chem. Soc.* **2009**, *131*, 15982. (c) Tanaka, K.; Kitamura, N.; Naka, K.; Chujo, Y. *Chem. Commun.* **2008**, 6176.
(9) (a) Vutukuri, D. R.; Basu, S.; Thayumanavan, S. *J. Am. Chem. Soc.* **2004**, *126*, 15636. (b) Aathimanikandan, S. V.; Savariar, E. N.; Thayumanavan, S. *J. Am. Chem. Soc.* **2005**, *127*, 14922. (c) Azagarsamy, M. A.; Sokkalingam, P.; Thayumanavan, S. *J. Am. Chem. Soc.* **2009**, *131*, 14184. (d) Azagarsamy, M. A.; Yesilyurt, V.; Thayumanavan, S. *J. Am. Chem. Soc.* **2010**, *132*, 4550. (e) Yesilyurt, V.; Ramireddy, R.; Thayumanavan, S. *Angew. Chem., Int. Ed. Engl.* **2011**, *50*, 3038. (f) Raghupathi, K. R.; Guo, J.; Munkhbat, O.; Rangadurai, P.; Thayumanavan, S. *Acc. Chem. Res.* **2014**, *47*, 2200.
(10) Bloembergen, N.; Purcell, E. M.; Pound, R. V. *Phys. Rev.* **1948**, *73*, 679.
(11) Harnoy, A. J.; Rosenbaum, I.; Tirosh, E.; Ebenstein, Y.; Shaharabani, R.; Beck, R.; Amir, R. J. *J. Am. Chem. Soc.* **2014**, *136*, 7531.
(12) (a) Grayson, S. M.; Fréchet, J. M. J. *Chem. Rev.* **2001**, *101*, 3819. (b) Tomalia, D. A.; Fréchet, J. M. J. *J. Polym. Sci. A, Polym. Chem.* **2002**, *40*, 2719. (c) Svenson, S.; Tomalia, D. A. *Adv. Drug Delivery Rev.* **2005**, *57*, 2106. (d) Newkome, G. R.; He, E.; Moorefield, C. N. *Chem. Rev.* **1999**, *99*, 1689. (e) Bosman, A. W.; Janssen, H. M.; Meijer, E. W. *Chem. Rev.* **1999**, *99*, 1665. (f) Caminade, A. M.; Majoral, J. P. *Acc. Chem. Res.* **2004**, *37*, 341. (g) Astruc, D.; Boisselier, E.; Ornelas, C. *Chem. Rev.* **2010**, *110*, 1857.
(13) (a) Newkome, G. R.; Yao, Z.; Baker, G. R.; Gupta, V. K. *J. Org. Chem.* **1985**, *50*, 2003. (b) Hawker, C. J.; Wooley, K. L.; Fréchet, J. M. J. *J. Chem. Soc., Perkin Trans. 1* **1993**, 1287. (c) Tomalia, D. A.; Baker, H.; Dewald, J.; Hall, M.; Kallos, G.; Martin, S.; Roeck, J.; Ryder, J.; Smith, P. *Polym. J.* **1985**, *17*, 117. (d) Azagarsamy, M. A.; Sokkalingam, P.; Thayumanavan, S. *J. Am. Chem. Soc.* **2009**, *131*, 14184.
(14) (a) Bo, Z.; Schäfer, A.; Franke, P.; Schlüter, a. D. *Org. Lett.* **2000**, *2*, 1645. (b) Azagarsamy, M. A.; Krishnamoorthy, K.; Sivanandan, K.; Thayumanavan, S. *J. Org. Chem.* **2009**, *74*, 9475. (c) Sivanandan, K.; Aathimanikandan, S. V.; Arges, C. G.; Bardeen, C. J.; Thayumanavan, S. *J. Am. Chem. Soc.* **2005**, *127*, 2020. (d) Sivanandan, K.; Sandanaraj, B. S.; Thayumanavan, S. *J. Org. Chem.* **2004**, *69*, 2937. (e) Lee, C.; Lo, S.-T.; Lim, J.; da Costa, V. C. P.; Ramezani, S.; Öz, O. K.; Pavan, G. M.; Annunziata, O.; Sun, X.; Simanek, E. E. *Mol. Pharmaceutics* **2013**, *10*, 4452. (f) Woller, E. K.; Cloninger, M. J. *Biomacromolecules* **2001**, *2*, 1052.
(15) Fuller, J. M.; Raghupathi, K. R.; Ramireddy, R. R.; Subrahmanyam, A. V.; Yesilyurt, V.; Thayumanavan, S. *J. Am. Chem. Soc.* **2013**, *135*, 8947.

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