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Combined A^3 Coupling and Click Chemistry Approach for the Synthesis of Dendrimer-Based Biological Tools

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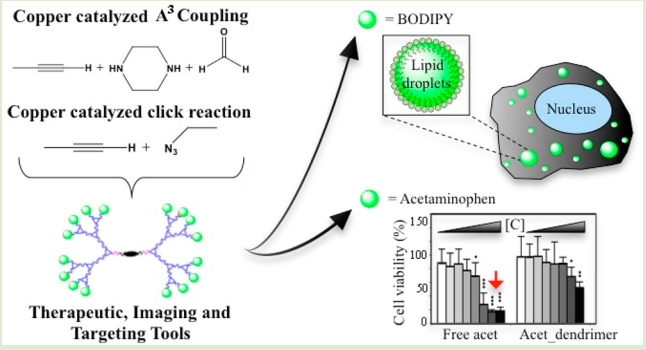
Supporting Information

ABSTRACT: We report a versatile approach in which two highly efficient chemical reactions, multicomponent A^3 coupling and alkyne−azide click chemistry, are combined to construct dendrimer-based tools for applications in biology. Using a convergent approach, dendrons with desired architecture and an alkyne at the focal point are first assembled and then stitched together via multicomponent A^3 coupling reaction. The desired functional groups, including a stealth agent, imaging dye, and drug molecules, could be easily covalently linked to the surfaces of these hyperbranched macromolecules using alkyne−azide click chemistry. These A^3-click dendrimers are noncytotoxic at concentrations as high as 1 μM and in fact reduce the toxicity of the drug. The dye-coated dendrimers specifically target and localize in lipid droplets. This unison methodology represents an attractive chemical strategy in exploiting the untapped potential of A^3 coupling and facilitating the development of nanodevices for imaging and drug delivery.



Dendrimers are hyperbranched macromolecules with a wide range of demonstrated applications in materials, catalysis, and biology.[1] Since their inception in the 1980s, the construction of these unique architectures has seen remarkable development.[2] A large variety of methodologies have been employed for their synthesis, and the inclusion of highly efficient click reactions, specifically copper(I)-catalyzed alkyne−azide (CuAAC) coupling,[3] is seen as a major breakthrough in dendrimer synthesis in the past decade.[4] This technique has been used in both convergent and divergent synthesis to grow the dendrimer generations, link dendrons together, and decorate their peripheries with a variety of functional groups.[5] The major advantages click reactions afford include high yields, byproduct free chemical transformations, and tolerance to a variety of solvents and functional groups. With the increasing potential for the use of dendrimers in a variety of applications, it will be advantageous to develop novel, efficient, and greener synthetic reactions comparable to click chemistry to build these macromolecular architectures. In this regard, highly efficient multicomponent reactions[6] that could combine three or more reaction partners in one pot and yield complex products from simple starting materials offer an exciting platform. A^3 coupling, first reported by Li's group, is one such example of a chemical reaction which is atom-economic and environmentally friendly.[7] A^3 coupling is a three-component catalytic coupling reaction of an alkyne, aldehyde, and amine to yield a propargyl amine. This three-component one-pot reaction combines the selectivity of reductive alkylation and the advantage of alkylation in a single step, under mild conditions. A wide variety of transition metal catalysts can be used to achieve A^3 coupling, including silver, gold, ruthenium/copper, copper, cobalt, iron, indium, and iridium.[8]

We envision that A^3 coupling has all the desired features to be used as a synthetic tool for dendrimer build up, in combination with click chemistry. To the best of our knowledge, A^3 coupling has never been employed before for dendrimer synthesis. We report here a highly facile synthetic methodology to construct dendrimers via a combination of A^3 coupling and CuAAC click reaction. The synthesis was achieved using convergent methodology in which the dendrons were constructed using click chemistry and were then stitched to the core via A^3 coupling reaction. The surface of A^3-click dendrimers can be decorated with a variety of biologically active functional groups. Dendrimer-conjugated NSAIDs showed improved solubility and bioavailability.[1c,d] We selected acetaminophen as a functional group with anti-inflammatory properties. The rationale for selecting acetaminophen was that it is an anti-inflammatory drug, which requires an appropriate

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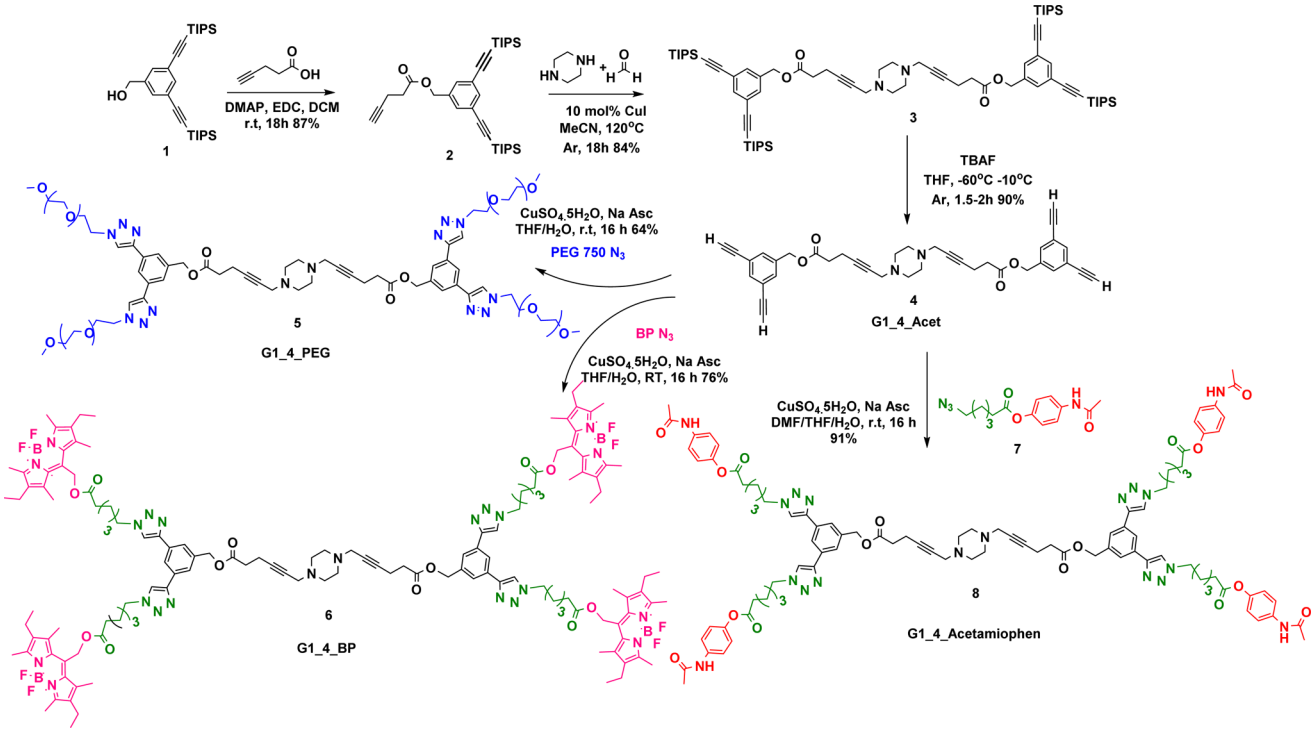
Scheme 1. Synthesis of G1 Dendrimer 4 with Free Acetylenes and Dendrimers 5, 6, and 8 with Surface Decorated with PEG, BODIPY, and Acetaminophen, Respectively



means of delivery to avoid or at least reduce its adverse side effects (e.g., gastrointestinal and renal). Inflammation is associated with an enhanced expression and release of pro-inflammatory cytokines and changes in morphology and function of cellular organelles. The formation of lipid droplets, dynamic organelles strongly responding to pro-inflammatory stimuli in macrophages and microglia, has been reported by various groups.[4c,9]

To demonstrate the intracellular targeting capabilities of A^3-click dendrimers to lipid droplets, the imaging dye BODIPY 493/503 was covalently bound to provide a stable lipid droplet probe. BODIPY 493/503 is a well-characterized neutral lipid label. Chemical modifications of A^3-click dendrimers with anti-inflammatory drugs and fluorescent labels could provide new ways of targeting lipid droplets to investigate their role in pathologies associated with inflammation.

We adopted a convergent approach to synthesize dendrimers using a combination of click and A^3 coupling chemistry. The dendrons were first synthesized using CuAAC click reaction and then joined together using a three-component A^3 coupling reaction to yield the piperazine-based core. The construction of a G1 dendrimer was initiated from benzyl alcohol **1** with triisopropylsilyl-protected acetylenes (Scheme 1). This molecule was synthesized by an adaptation of our previously published procedure.[10] The hydroxyl group on **1** was utilized to perform Steglich esterification[11] reaction with pentynoic acid to give compound **2** which has an acetylene focal point (one of the components of A^3 coupling reaction) and two protected alkynes to further participate in click reaction. A^3 coupling was performed by combining the alkyne on **2**, formaldehyde, and piperazine in one pot, using a catalytic amount of copper iodide. The reaction proceeded smoothly to afford G1 dendrimer **3** with four protected alkynes in a good yield. Triisopropylsilyl (TIPS) groups on **3** were subsequently removed using *tert*-butyl ammonium fluoride (TBAF) to yield dendrimer **4** with free acetylenes at the periphery. The TIPS deprotection was a straightforward reaction, and the product was purified by washing with diethyl ether several times without any column purification. The synthesis was monitored by ^{1}H NMR, which clearly indicated the disappearance of TIPS protons at 1.12 ppm and the appearance of acetylene protons at 3.11 ppm. It is worth mentioning here that the conditions for TIPS deprotection play a significant role in the overall yield of the product. The use of excess amounts of TBAF, increasing reaction temperature, or reaction time leads to cleavage of the ester linkages in the dendrimer backbone, with the appearance of multiple spots on a thin-layer chromatogram (TLC). The free acetylene groups on the surface of dendrimer **4** were reacted with PEG_{750}azide[12] using click conditions to obtain surface PEGylated dendrimer **5**. The disappearance of alkyne protons and the appearance of PEG protons in ^{1}H NMR spectra were used to monitor the progress of the reaction. In order to attach the imaging agent BODIPY dye on the periphery of dendrimer **4**, BODIPY−azide[1a] was clicked on **4**, to afford dendrimer **6** decorated with four BODIPY molecules. Dendrimer **4** was further utilized to append active pharmaceutical agents on its surface. For this purpose, an azide group was generated on acetaminophen by carrying out an esterification reaction using the phenolic−OH on the drug molecule and carboxylic acid of 6-azidohexanoic acid to afford compound **7**. Dendrimer **4** was reacted with compound **7** to give the dendrimer−drug conjugate **8**. The complete disappearance of the signal for acetylene protons and the emergence of peaks for acetaminophen protons in the ^{1}H NMR spectrum clearly indicated the formation of the product.

In order to evaluate the efficacy of this synthetic methodology, second and third generation A^3-click dendrimers were also synthesized. The G2 dendron **11** was first constructed

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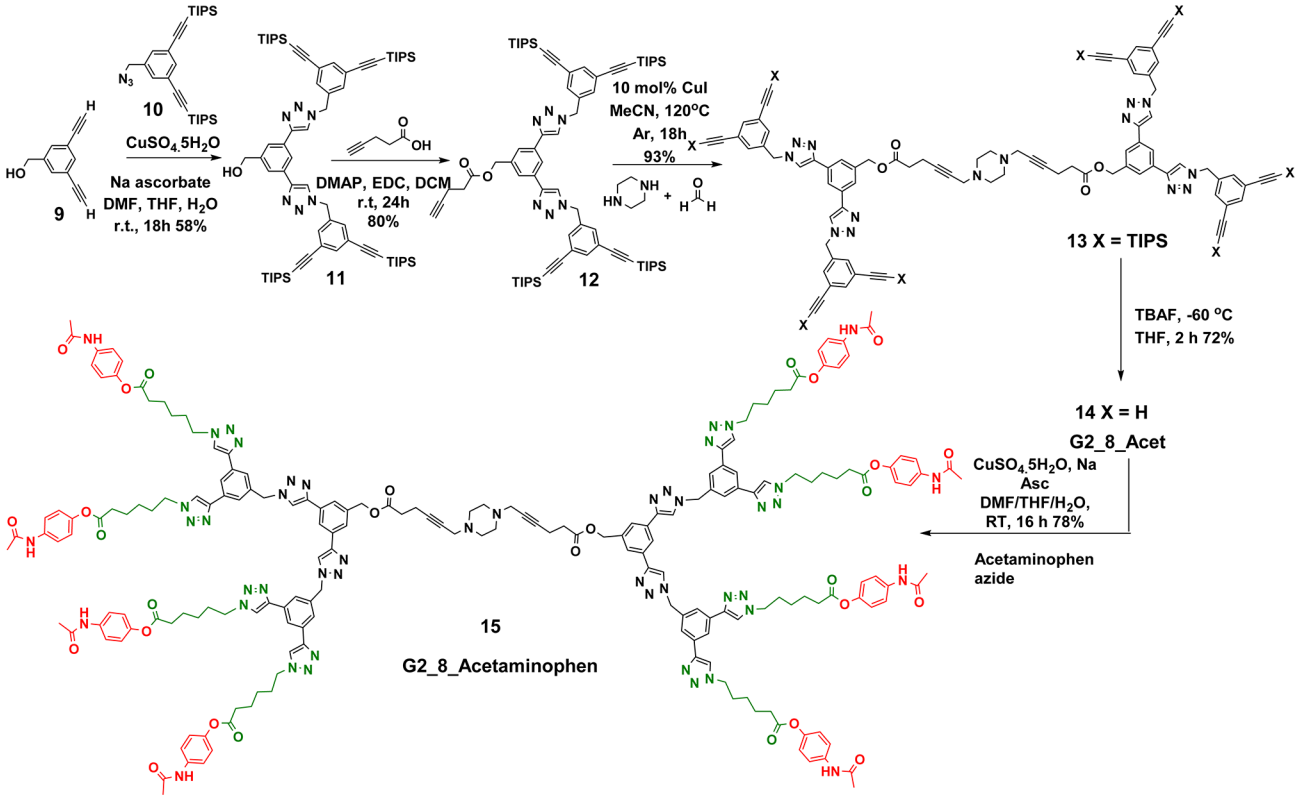
Scheme 2. Schematic Representation for the Synthesis of Second Generation A^3-Click Dendrimer 14 and Its Surface Functionalization with Acetaminophen Molecules

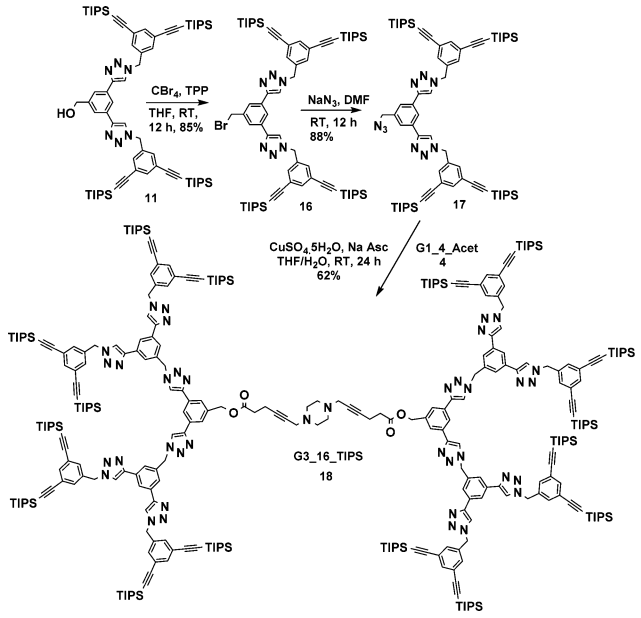


from building blocks **9** and **10** using click chemistry (Scheme 2). The compounds **9** and **10** were synthesized by the adaptation of our previously reported procedure[10] and used in click reaction to yield dendron **11** with the benzyl alcohol focal point and four protected acetylenes in about 58% yield. The benzyl alcohol group in **11** was modified to have an acetylene group to participate as one of the components of A^3 coupling reaction. It was achieved by an esterification reaction of dendron **11** with pentynoic acid to yield **12**. A^3 coupling was then carried out using this dendron **12**, piperazine, and formaldehyde in the presence of catalytic amounts of copper iodide. It led to the synthesis of the G2 dendrimer **13** with eight protected alkyne terminal groups. The product was purified by preparative thin-layer chromatography (TLC). The acetylenes were subsequently deprotected using TBAF at −60 °C to afford dendrimer **14** with eight free alkynes at the periphery. The latter reaction time should not be allowed to go beyond 2 h to avoid the cleavage of ester bonds in the dendrimer backbone. The purification was very simple, and the product was obtained by carrying out multiple washings with diethyl ether without any column purification. Once again, the disappearance of TIPS protons and the appearance of alkyne protons in the ^{1}H NMR spectrum confirmed the product formation. The terminal alkyne groups in **14** were utilized to attach acetaminophen molecules through click reaction with acetaminophen-azide **7** to obtain dendrimer−drug conjugate **15** containing eight drug molecules at its surface.

The synthetic elaboration to the G3 dendrimer was achieved by clicking the G2 dendron onto the A^3-coupled G1 core (Scheme 3).

For this purpose, the benzyl alcohol moiety in dendron **11** was converted to benzyl bromide (**16**), which was further

Scheme 3. Synthesis of Third Generation A^3-Click Dendrimer 18 with Sixteen Protected Acetylenes at the Surface



modified to an azide functional group (**17**). The dendron **17** was reacted with the G1 dendrimer via CuAAC reaction to yield G3 dendrimer **8** with sixteen protected acetylene groups on the surface. The latter can then be used to covalently link biologically active entities at its surface by deprotecting the acetylenes, followed by click reaction as presented earlier in Schemes 1 and 2.

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Biological experiments with A^3-click dendrimers were performed with the following aims: (1) to determine if the constructs are cytotoxic to macrophages within nanomolar and micromolar concentrations, and (2) to see if the fluorescent BODIPY 495/503 labeled dendrimers can detect lipid droplets in macrophages. To determine dendrimer safety in a biological system, we tested **G1_4_Acet** (**4**), **G2_8_Acet** (**14**), **G1_4_PEG** (**5**), and acetaminophen analogues (**8**, **15**) in J774A.1 murine macrophage cells. A concentration-dependent decrease in cell viability was established for the compounds within 1−500 μM concentrations. Following 24 h exposure to these compounds, mitochondrial metabolic activity was measured by absorbance from formazan crystals dissolved in DMSO (Supporting Information, Figure 40). The results show no marked mitochondrial impairments in cells exposed to A^3-click dendrimers up to 1 μM in concentration. In parallel experiments, a cell number determination was performed using high throughput imaging (Operetta) and cell counting from fluorescent micrographs in order to reveal possible loss of macrophages exposed to high dendrimer concentrations. Data for the treatments with dendrimers ranging from 1 nM to 500 μM for 24 h are shown in Figure 1.

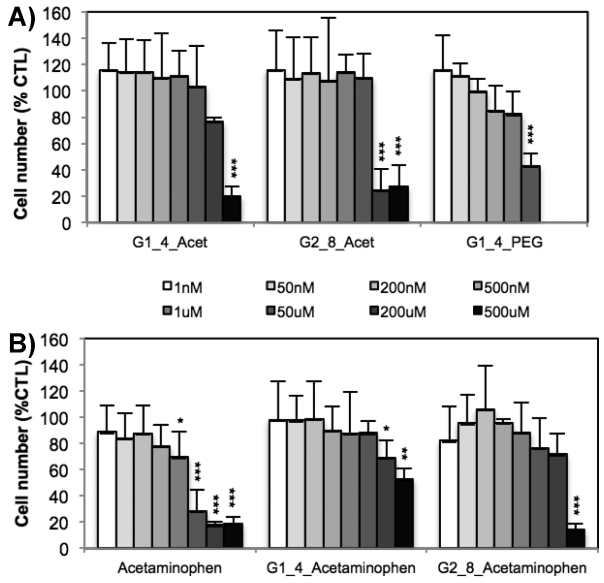

Figure 1. Viability of J774A.1 macrophages treated with (A) **G1_4_Acet** (**4**), **G2_8_Acet** (**14**), **G1_4_PEG** (**5**), (B) Acetaminophen, **G1_4_Acetaminophen** (**8**), and **G2_8_Acetaminophen** (**15**) represented by * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ as calculated using a t test.

In agreement with the results for mitochondrial activity, the compounds did not elicit a decrease in total cell number in concentrations up to 1 μM. In contrast, acetaminophen in its free form (i.e., not bound to a dendrimer carrier) began to show significant toxicity at this concentration. Further, the dendrimers seemed to dampen the toxicity associated with free acetaminophen, as seen by the reduced loss of cells as compared to the drug alone. At very high concentrations (200−500 μM) all compounds reduced cell survival presumably because macrophages are loaded beyond their abilities to effectively eliminate the compounds.

Follow up experiments were sought to demonstrate the versatility of A^3-click dendrimers by exploiting them for imaging and targeting of intracellular organelles. Lipid droplets are dynamic organelles that change in composition in conditions of stress and inflammation.[3,4d,5a,13] Lipid droplets were induced in macrophages by the addition of the unsaturated fatty acid, oleic acid (50 μM, 6 h). After LD induction, **G1_4_BP** (**6**) was incubated in cells for 5, 10, or 30 min. At treatment end-time points, cells were washed, fixed, and labeled with the lipid-droplet specific dye LipidTOX. The imaging studies demonstrate that **G1_4_BP** is highly permeable to cells and quickly localizes to lipid droplets; they were detectable after 5 min (green) and colocalized in part with red LipidTOX. Cells were treated with LipidTOX or **G1_4_BP** independently and imaged in all channels to eliminate artifacts due to bleed through (Supporting Information, Figure 41). Imaging of cells without OA treatment further demonstrates that in the absence of lipid droplets **G1_4_BP** does not localize to other organelles but rather remains diffuse within the cells (Supporting Information Figure 42). Interestingly, as seen in Figure 2 after 30 min, the tagged dendrimers may be

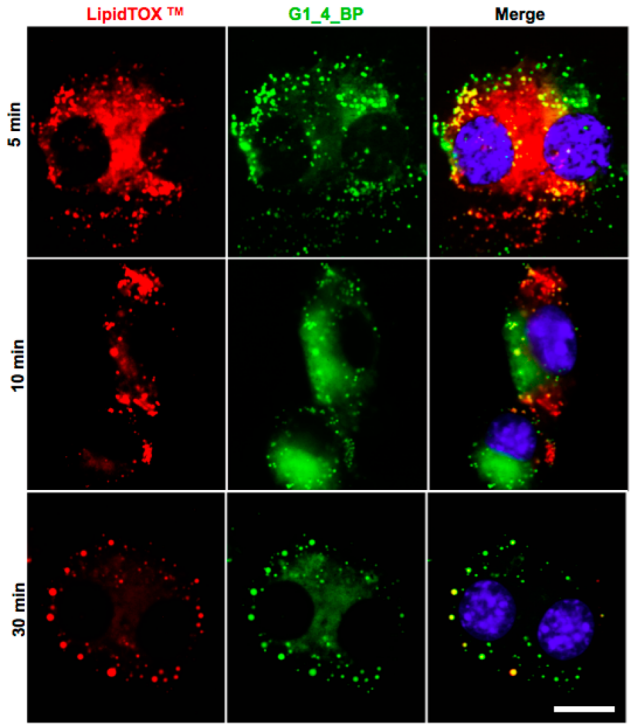

Figure 2. Murine macrophages treated with **G1_4_BP** (**6**) (green) for 5, 10, or 30 min quickly take up the dendrimer, which localizes with a population of lipid droplets labeled with LipidTOX (red). Nuclei are labeled with Hoechst 33342 (10 μM, 10 min). Scale bar represents 10 μm.

preferentially localizing to a subset of lipid droplets as indicated by the incomplete colocalization with LipidTOX. This is in contrast to free BODIPY493/503 which shows almost perfect superimposition when compared to LipidTOX (Supporting Information Figure 43).

Fluorescent BODIPY dyes such as 493/503 partition to the lipid body core and are used for labeling lipid droplets within size ranges detectable by optical microscopes. Our data clearly show several populations of these organelles distinguishable by their sizes and location. Future studies should explore lipid composition and proteins associated with the surface of the lipid droplets preferentially targeted by **G1_4_BP**. Thus, a **G1_4_BP** probe could be a useful marker to discern specific

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LD populations detectable upon macrophage stimulation with pro-survival (e.g., trophic factors, oleic acid) and noxious stimuli.

In conclusion, this study exemplifies the unexplored potential of the multicomponent A^3 coupling reaction in expanding the scope of dendrimer synthesis. Upon combining with click chemistry, it provides a simple and efficient strategy in constructing functionalized dendrimers as versatile tools for imaging and drug delivery. The A^3-click dendrimers are safe in concentrations up to 1 μM and do not cause any marked deleterious effect to macrophages. These functionalized dendrimers accumulate in lipid droplets, and the versatility of this methodology provides an advantageous platform in developing dendrimer-based nanocarriers and probes for these organelles.

■ ASSOCIATED CONTENT

Supporting Information

Experimental and synthesis details and NMR spectra of the compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) (a) Hourani, R.; Jain, M.; Maysinger, D.; Kakkar, A. *Chem.—Eur. J.* **2010**, *16*, 6164−6168. (b) Mather, B. D.; Viswanathan, K.; Miller, K. M.; Long, T. E. *Prog. Polym. Sci.* **2006**, *31*, 487−531. (c) Cheng, Y.; Man, N; Xu, T.; Fu, R; Wang, X; Wen, L. *J. Pharm. Sci.* **2007**, *96*, 595−602. (d) Svenson, S. *Eur. J. Pharm. Biopharm.* **2009**, *71*, 445−462. (e) Khandare, J.; Calderon, M.; Dagia, N. M.; Haag, R. *Chem. Soc. Rev.* **2012**, *41*, 2824−2848. (f) Hourani, R.; Kakkar, A. *Macromol. Rapid Commun.* **2010**, *31*, 947−974. (g) Tomalia, D. A.; Fréchet, J. M. J. *J. Polym. Sci., Part A: Polym. Chem.* **2002**, *40*, 2719−2728.

(2) (a) Buhleier, E.; Wehner, W.; Vogtle, F. *Synthesis* **1978**, *1978*, 155−158. (b) Tomalia, D. A.; Baker, H.; Dewald, J.; Hall, M.; Kallos, G.; Martin, S.; Roeck, J.; Ryder, J.; Smith, P. *Polym. J. (Tokyo)* **1985**, *17*, 117−132.

(3) Khatchadourian, A.; Maysinger, D. *Mol. Pharmaceutics* **2009**, *6*, 1125−1137.

(4) (a) Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2001**, *40*, 2004−2021. (b) Franc, G.; Kakkar, A. K. *Chem. Soc. Rev.* **2010**, *39*, 1536−1544. (c) Khatchadourian, A.; Bourque, S. D.; Richard, V. R.; Titorenko, V. I.; Maysinger, D. *Biochim. Biophys. Acta, Mol. Cell Biol. Lipids* **2012**, *1821*, 607−617. (d) Suzuki, M.; Shinohara, Y.; Ohsaki, Y.; Fujimoto, T. *J. Electron Microsc.* **2011**, *60*, S101−S116. (e) Sharma, A.; Neibert, K.; Sharma, R.; Hourani, R.; Maysinger, D.; Kakkar, A. *Macromolecules* **2011**, *44*, 521−529.

(5) (a) Melo, R. C. N.; D'Avila, H.; Wan, H.-C.; Bozza, P. T.; Dvorak, A. M.; Weller, P. F. *J. Histochem. Cytochem.* **2011**, *59*, 540−556. (b) Xi, W.; Scott, T. F.; Kloxin, C. J.; Bowman, C. N. *Adv. Funct. Mater.* **2014**, *24*, 2572−2590.

(6) (a) Karimi, B.; Gholinejad, M.; Khorasani, M. *Chem. Commun.* **2012**, *48*, 8961−8963. (b) Dömling, A.; Wang, W.; Wang, K. *Chem. Rev.* **2012**, *112*, 3083−3135. (c) Ruijter, E.; Scheffelaar, R.; Orru, R. V. A. *Angew. Chem., Int. Ed.* **2011**, *50*, 6234−6246. (d) Zani, L.; Bolm, C. *Chem. Commun.* **2006**, 4263−4275.

(7) Wei, C.; Li, C.-J. *J. Am. Chem. Soc.* **2003**, *125*, 9584−9585.

(8) (a) Wei, C.; Li, Z.; Li, C.-J. *Synlett* **2004**, *2004*, 1472−1483. (b) Uhlig, N.; Li, C.-J. *Org. Lett.* **2012**, *14*, 3000−3003. (c) Zeng, T.; Chen, W.-W.; Cirtiu, C. M.; Moores, A.; Song, G.; Li, C.-J. *Green Chem.* **2010**, *12*, 570−573. (d) Chen, W.-W.; Bi, H.-P.; Li, C.-J. *Synlett* **2010**, *2010*, 475−479. (e) Bonfield, E. R.; Li, C.-J. *Adv. Synth. Catal.* **2008**, *350*, 370−374. (f) Yoo, W.-J.; Zhao, L.; Li, C.-J. *Aldrichimica Acta* **2011**, *44*, 43−51.

(9) (a) Martin, S.; Parton, R. G. *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 373−378. (b) Bozza, P. T.; Viola, J. P. B. *Prostaglandins, Leukotrienes Essent. Fatty Acids* **2010**, *82*, 243−250. (c) Dugail, I. *Biochimie* **2014**, *96*, 102−105.

(10) Hourani, R.; Sharma, A.; Kakkar, A. *Tetrahedron Lett.* **2010**, *51*, 3792−3795.

(11) Neises, B.; Steglich, W. *Angew. Chem., Int. Ed.* **1978**, *17*, 522−524.

(12) Sharma, A.; Khatchadourian, A.; Khanna, K.; Sharma, R.; Kakkar, A.; Maysinger, D. *Biomaterials* **2011**, *32*, 1419−1429.

(13) Ohsaki, Y.; Suzuki, M.; Fujimoto, T. *Chem. Biol.* **2014**, *21*, 86−96.