# Labeling of Polymer Nanostructures for Medical Imaging: Importance of Cross-Linking Extent, Spacer Length, and Charge Density

**Jinqi Xu,[†,#] Guorong Sun,[†,#] Raffaella Rossin,[‡,#] Aviv Hagooly,[‡,#] Zicheng Li,[†] Ken-ichi Fukukawa,[§] Benjamin W. Messmore,[§] Dennis A. Moore,[⊥] Michael J. Welch,[†,‡] Craig J. Hawker,[§] and Karen L. Wooley*[,†,‡]**

*Center for Materials Innovation and Department of Chemistry, Washington University in Saint Louis, Saint Louis, Missouri 63130; Mallinckrodt Institute of Radiology, Washington University School of Medicine, Saint Louis, Missouri 63110; Materials Research Laboratory, University of California at Santa Barbara, Santa Barbara, California 93106; and Mallinckrodt Inc., 675 McDonnell Blvd., P.O. Box 5840, Saint Louis, Missouri 63134*

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Nanoscale architectures,[1] including spheres, cylinders, toroids, vesicles, disks, and other morphologies, have attracted much attention. Such nanoobjects with diverse properties have promise for many aspects of nanomedicine,[2] for instance, as carriers to deliver therapeutic agents to specific targets with release of their payloads in a controlled fashion. Great efforts have been devoted to spherical micelles,[3] which derive from amphiphilic block copolymers. Above the critical micelle concentration (cmc), micelles are thermodynamically stable in aqueous environments and are able to encapsulate hydrophobic drug molecules having poor water solubility. Meanwhile, their hydrophilic shell layer stabilizes the entire nanoobject and provides sites for addition of functional units. However, the stability of such nanoscale systems in vivo is of concern. To eliminate cmc restrictions, shell cross-linked nanoparticles (SCKs), a form of covalently stabilized micelles, have been developed by performing intramicellar cross-linking throughout the shell layer.[4]

The modular nature of SCKs allows their tailoring for specific applications and for the targeted drug delivery to and reporting of biological activities at specific sites. SCK nanoparticles have been further functionalized via various methodologies to provide surface-accessible, biologically active ligands.[5] The conjugation of various ligands onto pre-established nanoparticles has proved to be a versatile and straightforward method that allows for multiple numbers and types of ligands to be attached onto a well-defined scaffold.[6] In the case of SCKs, several chemistries have been explored for shell functionalization, including amidation[7] and click chemistry.[8] While in most cases the couplings were effective, the complexity of the systems makes general statements about coupling difficult. As this entire field of study involving the interactions between synthetic nanomaterials and biological systems relies upon efficient functionalization strategies, it is important to acquire a better understanding of the coupling chemistry and interactions between targeting ligands and polymeric nanoparticles.

* Corresponding author: Tel 314-9357136; Fax 314-9359844; e-mail klwooley@artsci.wustl.edu.
[†] Washington University in Saint Louis.
[‡] Washington University School of Medicine.
[§] University of California at Santa Barbara.
[⊥] Mallinckrodt Inc.
[#] These authors contributed equally to this work.

Radiolabeling of chelators conjugated onto nanostructures provides materials for in vivo imaging and also offers a highly sensitive quantitative measurement of the amount of accessible chelator and, therefore, can be used as a tool to determine the yield of conjugation chemistries. Herein, we report the conjugation of SCK nanoparticles with two types of DOTA (1,4,7,10-tetraazocyclododecane-*N*,*N*′,*N*″,*N*‴-tetraacetic acid) derivatives having different features, DOTAamine and DOTAlysine (Scheme 1). DOTA is a macrocyclic chelating agent widely used to chelate metal ions for diagnostic and therapeutic applications. Among these metals, copper-64 ($^{64}$Cu) has been investigated for its applications in both positron emission tomography (PET) and radiotherapy.[9] In this study, the $^{64}$Cu-labeling of DOTA-SCK conjugates is employed to provide new insights into the coupling chemistry, design of ligands, and transformation of SCKs into functional materials.

Several SCK samples with varying extents of cross-linking, **SCK1**−**SCK8**, were prepared from the self-assembly of amphiphilic diblock copolymers, poly(acrylic acid)-*b*-polystyrene (PAA-*b*-PS), with varying block lengths from 30 to 136 repeat units, followed by chemical cross-linking throughout the shell layer by reaction with 2,2′-(ethylenedioxy)-bis(ethylamine). These SCK nanoparticles had diameters that were in agreement with the sizes expected on the basis of the relative hydrophilic:hydrophobic balance and overall polymer chain lengths (Table 1).

Conjugation of each sample from **SCK1** to **SCK8** with DOTAamine molecules was performed via amidation chemistry, using *N*-hydroxysulfosuccinimide (sulfo-NHS)[10] in aqueous media (Scheme 1). The 50% cross-linked SCKs, **SCK5** to **SCK8**, were coupled with DOTAlysine, under similar experimental conditions. Consistent with previous observations,[7] DLS measurements of these conjugates presented similar hydrodynamic diameters to those of their respective SCK precursors. Each DOTAamine−SCK conjugate and DOTAlysine−SCK conjugate was subjected to $^{64}$Cu radiolabeling tests, under the same experimental protocols and in the same time frame. The specific activity of each DOTA−SCK conjugate was determined from the percent labeling efficiency, and the number of $^{64}$Cu-accessible DOTAs per DOTA-SCK was also obtained via a modified isotopic dilution method (Table 2).[7c,11]

The radiolabeling results indicated significant differences between the coupling yields of the DOTAamine and DOTAlysine compounds to the SCKs. It is obvious that the coupling efficiency of DOTAlysine with any of **SCK5** to **SCK8** was so low that both the specific activity ($\mu$Ci/$\mu$g) and the $^{64}$Cu-accessible DOTA per DOTA−SCK conjugate were much less than unity. In contrast, attachment of DOTAamine to **SCK5**−**SCK8** gave products that showed high levels of radiolabeling, i.e., 2−11 $\mu$Ci/$\mu$g of specific activity. Steric hindrance factors were first considered between a SCK nanoparticle having ca. 14−44 nm diameter with the DOTA moiety that is a macrocyclic molecule with a diameter no greater than 1 nm and a short spacer length. The nine-atom spacer between the reactive amino end of DOTAamine with its cyclen ring nitrogen atom was longer than that of DOTAlysine, having five atoms (Figure 1), resulting in a distinct enhancement of the coupling by DOTAamine. It has also been found that the coupling efficiency was low (ca. < 5%) between a maleimide derivative bearing an ethylene spacer and SCKs under the same experimental conditions.[12a] However, the negative zeta potential values of **SCK5** to **SCK8** (Table 1) indicated that the nanoparticle surface presented a high

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**Scheme 1. Conjugation Chemistry of SCK Nanoparticles with DOTAamine or DOTAlysine Molecules in Aqueous Media**

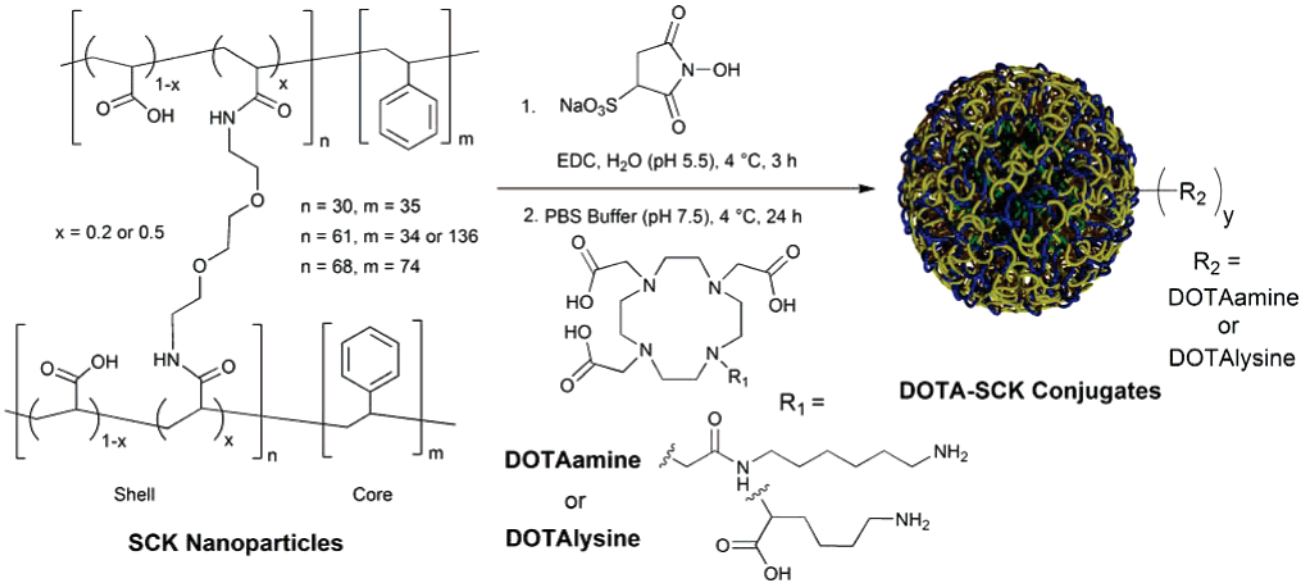


**Table 1. Physical Properties of Original SCKs**

| particle[a] | extents of cross-linking (%)[b] | $PAA_n$-b-$PS_m$[c] precursor | $n$ | $m$ | $(D_h)_n$ (nm, DLS)[d] | $D_{av}$ (nm, TEM)[e] | $H_{av}$ (nm, AFM)[f] | $N_{aggr}$[g] | $\zeta$/mV[h] |
|---|---|---|---|---|---|---|---|---|---|
| **SCK1** | 20 | **2** | 30 | 35 | 17 ± 4 | 15 ± 1 | 11 ± 2 | 307 | −11 ± 2 |
| **SCK2** | | **4** | 61 | 34 | 14 ± 5 | 11 ± 1 | 3 ± 1 | 125 | −13 ± 2 |
| **SCK3** | | **6** | 68 | 74 | 24 ± 4 | 17 ± 1 | 11 ± 1 | 211 | −11 ± 2 |
| **SCK4** | | **8** | 61 | 136 | 44 ± 6 | 25 ± 1 | 21 ± 2 | 324 | −10 ± 2 |
| **SCK5** | 50 | **2** | 30 | 35 | 16 ± 3 | 17 ± 1 | 12 ± 1 | 307 | −25 ± 2 |
| **SCK6** | | **4** | 61 | 34 | 14 ± 2 | 11 ± 1 | 5 ± 1 | 125 | −19 ± 1 |
| **SCK7** | | **6** | 68 | 74 | 22 ± 1 | 17 ± 1 | 14 ± 1 | 211 | −15 ± 1 |
| **SCK8** | | **8** | 61 | 136 | 34 ± 4 | 24 ± 2 | 20 ± 2 | 324 | −29 ± 1 |

[a] Sample concentrations were 0.20−0.30 mg/mL. [b] These values are based on the stoichiometry used during the cross-linking reaction. [c] Composition of amphiphilic diblock copolymer employed for micelle formation; polymer precursor numbers were defined in the Supporting Information. [d] Number-average hydrodynamic diameters measured in nanopure $H_2O$. [e] $D_{av}$ were measured for the SCK core. [f] Average heights measured by tapping-mode AFM. [g] Each aggregation number was calculated based on the particle core diameter from TEM and the contents of PS. [h] Zeta potential values measured in 5 mM PBS buffer (pH 7.4).

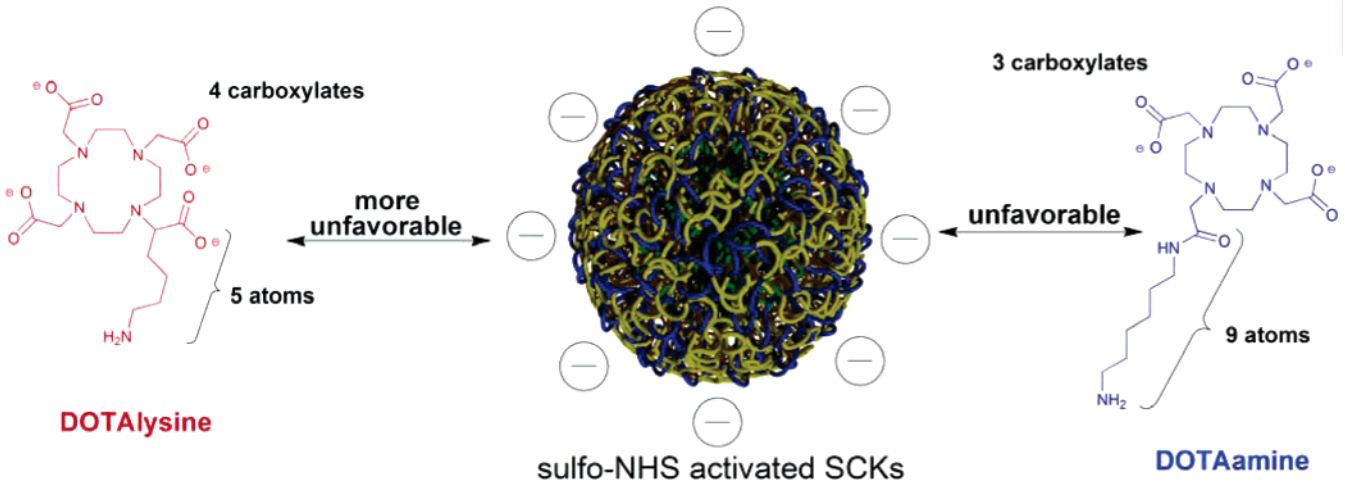


**Figure 1.** Structural comparisons of DOTAlysine with DOTAamine and sulfo-NHS activated SCK nanoparticles in PBS buffer (pH 7.5).

density of negatively charged carboxylates,[12b] while DOTAlysine and DOTAamine also contain carboxylates (Figure 1). The addition of more salt to the reaction mixture had little influence on the overall radiolabeling yield, implying that electrostatic repulsions between the SCKs and DOTA derivatives exerted minor influences on the coupling. Taken in combination, these results confirm that it is essential that a lengthy, inert spacer exists on the designed functionalities to minimize these steric and electrostatic hindrances, improving the reaction efficiency.

Each DOTAamine−SCK conjugate prepared from 20% cross-linked SCKs exhibited ca. 33−440% higher specific activity and increased numbers of $^{64}$Cu-accessible DOTAs per DOTA−SCK conjugate than those observed from the corresponding 50% cross-linked SCKs, derived from the same diblock copolymers (Table 2). For instance, DOTAamine−**SCK2** had 45 $\mu$Ci/$\mu$g, 3-fold greater than the 11 $\mu$Ci/$\mu$g of specific activity observed for DOTAamine−**SCK6**. Such remarkable improvement can be attributed to the fact that higher numbers of acrylic acid

**Table 2. $^{64}$Cu Radiolabeling Results of DOTA−SCK Conjugates[a]**

| functionalized particles | specific activity ($\mu$Ci $\mu$g$^{-1}$) | $^{64}$Cu-accessible DOTA per DOTA−SCK |
|---|---|---|
| DOTAamine−**SCK1** | 32 | 22 |
| DOTAamine−**SCK2** | 45 | 14 |
| DOTAamine−**SCK3** | 14 | 11 |
| DOTAamine−**SCK4** | 2 | 3 |
| DOTAamine−**SCK5** | 6 | 2 |
| DOTAamine−**SCK6** | 11 | 2 |
| DOTAamine−**SCK7** | 3 | <1 |
| DOTAamine−**SCK8** | 2 | <1 |

[a] Specific activities ($\mu$Ci $\mu$g$^{-1}$) and $^{64}$Cu-accessible DOTA per SCK of DOTAlysine−**SCK5** to DOTAlysine−**SCK8** conjugates were much less than unity.

residues were present on the surface of and within the subsurface of the SCKs with a lower degree of cross-linking, and those carboxylic acids were available for amidation with DOTAamine. Contributions from the larger number of carboxylic acids overcame the potential obstacles from steric factors and electrostatic repulsions (vide supra). Furthermore, it has been shown that the permeability of the cross-linked shell domains (membrane-type materials) is higher in SCKs with lower extents of cross-linking.[13] It is hypothesized that the permeability of the reagents within the shell affects the extent to which the coupling reactions can occur.

The overall structure and composition of the SCKs also affected the coupling chemistry with DOTAamine. The order of specific activity was DOTAamine−**SCK2** > DOTAamine−**SCK1** > DOTAamine−**SCK3** > DOTAamine−**SCK4**, where for each the degree of cross-linking was 20% (Table 2). **SCK2** to **SCK4** contained similar lengths of PAA segments, but incrementally greater lengths of hydrophobic, glassy PS segments, which decreased the hydrophilic:hydrophobic balance and resulted in increased aggregation numbers and particle sizes. **SCK2**, therefore, contained the greatest proportion of PAA content, followed by **SCK3** and then **SCK4**. The better performance of DOTAamine−**SCK2** suggests that the overall composition of the nanoparticles is an important parameter for surface/shell functionalization efficiencies. A similar argument is suggested to explain the differences observed between the radiolabeling of **SCK1** and **SCK2**, which possessed similar lengths for the PS block but different PAA block lengths.

In summary, we have demonstrated optimized conjugation of DOTA chelators onto SCK nanoparticles in aqueous media. The coupling efficiency, determined by $^{64}$Cu radiolabeling studies, suggests that the amidation chemistry depends on the nature of the surface and other physical properties of SCK nanoparticles, including charge density, permeability, extents of cross-linking, compositions of the domains within the SCKs, and their relative contribution to the overall structure. In addition, the chemical structure and composition of the DOTA chelator are also critically important for efficient reaction. These results can have a great influence on the design and functionalization of nanoparticles, broadening their applications in advanced drug delivery. Currently, the optimized number of $^{64}$Cu-accessible DOTA chelators per DOTA−SCK conjugate is ca. 22, leading to good signal-to-noise ratios in PET imaging. However, there is still a need for improvement to realize exceptionally high radiolabeling yields that will allow application of DOTA−SCKs as highly sensitive in vivo PET imaging agents and allow the minimum amount of imaging agent to be used. More efforts are being devoted to further improve the sensitivity and specificity of such conjugates.

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**Supporting Information Available:** Complete description of experimental details. This materials is available free of charge via the Internet at http://pubs.acs.org.

## References and Notes

(1) (a) Won, Y.-Y.; Davis, H. T.; Bates, F. S. *Science* **1999**, *283*, 960−963. (b) Discher, D. E.; Eisenberg, A. *Science* **2002**, *297*, 967−973. (c) Frechet, J. M. J. *J. Polym. Sci., Part A: Polym. Chem.* **2003**, *41*, 3713−3725. (d) Li, Z.; Kesselman, E.; Talmon, Y.; Hillmyer, M. A.; Lodge, T. P. *Science* **2004**, *306*, 98−101. (e) Pochan, D. J.; Chen, Z.; Cui, H.; Hales, K.; Qi, K.; Wooley, K. L. *Science* **2004**, *306*, 94−97. (f) Hawker, C. J.; Wooley, K. L. *Science* **2005**, *309*, 1200−1205.

(2) (a) Esfand, R.; Tomalia, D. A. *Drug Discovery Today* **2001**, *6*, 427−436. (b) Kabanov, A. V.; Lemieux, P.; Vinogradov, S.; Alakhov, V. *Adv. Drug Delivery Rev.* **2002**, *54*, 223−233. (c) Davis, M. E.; Brewster, M. E. *Nat. Rev. Drug Discovery* **2004**, *3*, 1023−1035. (d) Langer, R.; Tirrell, D. A. *Nature (London)* **2004**, *428*, 487−492. (e) Torchilin, V. P. *Nat. Rev. Drug Discovery* **2005**, *4*, 145−160. (f) Peppas, N. A.; Hilt, J. Z.; Khademhosseini, A.; Langer, R. *Adv. Mater.* **2006**, *18*, 1345−1360.

(3) (a) Allen, C.; Maysinger, D.; Eisenberg, A. *Colloids Surf., B* **1999**, *16*, 3−27. (b) Rösler, A.; Vandermeulen, G. W. M.; Klok, H.-A. *Adv. Drug Delivery Rev.* **2001**, *53*, 95−108. (c) Gaucher, G.; Dufresne, M.-H.; Sant, V. P.; Kang, N.; Maysinger, D.; Leroux, J.-C. *J. Controlled Release* **2005**, *109*, 169−188. (d) Nishiyama, N.; Kataoka, K. *Pharmacol. Ther.* **2006**, *112*, 630−648.

(4) (a) Thurmond, K. B., II; Kowalewski, T.; Wooley, K. L. *J. Am. Chem. Soc.* **1996**, *118*, 7239−7240. (b) O'Reilly, R. K.; Hawker, C. J.; Wooley, K. L. *Chem. Soc. Rev.* **2006**, *35*, 1068−1083.

(5) (a) Yasugi, K.; Nakamura, T.; Nagasaki, Y.; Kato, M.; Kataoka, K. *Macromolecules* **1999**, *32*, 8024−8032. (b) Bes, L.; Angot, S.; Limer, A.; Haddleton, D. M. *Macromolecules* **2003**, *36*, 2493−2499. (c) Joralemon, M. J.; Murthy, K. S.; Remsen, E. E.; Becker, M. L.; Wooley, K. L. *Biomacromolecules* **2004**, *5*, 903−913. (d) Qi, K.; Ma, Q.; Remsen, E. E.; Clark, C. G., Jr.; Wooley, K. L. *J. Am. Chem. Soc.* **2004**, *126*, 6599−6607. (e) Joralemon, M. J.; Smith, N. L.; Holowka, D.; Baird, B.; Wooley, K. L. *Bioconjugate Chem.* **2005**, *16*, 1246−1256. (f) Licciardi, M.; Tang, Y.; Billingham, N. C.; Armes, S. P.; Lewis, A. L. *Biomacromolecules* **2005**, *6*, 1085−1096.

(6) (a) Templeton, A. C.; Wuelfing, M. P.; Murray, R. W. *Acc. Chem. Res.* **2000**, *33*, 27−36. (b) Medintz, I. L.; Uyeda, H. T.; Goldman, E. R.; Mattoussi, H. *Nat. Mater.* **2005**, *4*, 435−446. (c) Bull, S. R.; Guler, M. O.; Bras, R. E.; Meade, T. J.; Stupp, S. I. *Nano Lett.* **2005**, *5*, 1−4. (d) Frankamp, B. L.; Fischer, N. O.; Hong, R.; Srivastava, S.; Rotello, V. M. *Chem. Mater.* **2006**, *18*, 956−959.

(7) (a) Becker, M. L.; Remsen, E. E.; Pan, D.; Wooley, K. L. *Bioconjugate Chem.* **2004**, *15*, 699−709. (b) Pan, D.; Turner, J. L.; Wooley, K. L. *Macromolecules* **2004**, *37*, 7109−7115. (c) Sun, X.; Rossin, R.; Turner, J. L.; Becker, M. L.; Joralemon, M. J.; Welch, M. J.; Wooley, K. L. *Biomacromolecules* **2005**, *6*, 2541−2554.

(8) (a) Joralemon, M. J.; O'Reilly, R. K.; Hawker, C. J.; Wooley, K. L. *J. Am. Chem. Soc.* **2005**, *127*, 16892−16899. (b) O'Reilly, R. K.; Joralemon, M. J.; Hawker, C. J.; Wooley, K. L. *J. Polym. Sci., Part A: Polym. Chem.* **2006**, *44*, 5203−5217.

(9) (a) Connett, J. M.; Anderson, C. J.; Guo, L.-W.; Schwarz, S. W.; Zinn, K. R.; Rogers, B. E.; Siegel, B. A.; Philpott, G. W.; Welch, M. J. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 6814−6818. (b) McCarthy, D. W.; Bass, L. A.; Cutler, P. D.; Shefer, R. E.; Klinkowstein, R. E.; Herrero, P.; Lewis, J. S.; Cutler, C. S.; Anderson, C. J.; Welch, M. J. *Nucl. Med. Biol.* **1999**, *26*, 351−358.

(10) Lewis, M. R.; Kao, J. Y.; Anderson, A. L. J.; Shively, J. E.; Raubitschek, A. *Bioconjugate Chem.* **2001**, *12*, 320−324.

(11) Anderson, C. J.; Connett, J. M.; Schwarz, S. W.; Rocque, P. A.; Guo, L. W.; Philpott, G. W.; Zinn, K. R.; Meares, C. F.; Welch, M. J. *J. Nucl. Med.* **1992**, *33*, 1685−1691.

(12) (a) Sun, G.; Wooley, K. L., unpublished results. (b) Ma, Q.; Remsen, E. E.; Kowalewski, T.; Wooley, K. L. *J. Am. Chem. Soc.* **2001**, *123*, 4627−4628.

(13) Murthy, S. K.; Ma, Q.; Clark, C. G., Jr.; Remsen, E. E.; Wooley, K. L. *Chem. Commun.* **2001**, 773−774.

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