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Fluorescein-Functionalized Silica Nanoparticles as a Selective Fluorogenic Chemosensor for Cu^{2+} in Living Cells

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Fluorescein-functionalized silica nanoparticles (**1**) were prepared by sol–gel reaction, and their optical sensing abilities were studied towards finding a new type of synthetic fluorogenic chemosensor for imaging Cu^{2+} ions in living cells. Interestingly, upon addition of Cu^{2+} in a H_2O suspension of **1** at pH 7.4, **1** displays large chelation-enhanced quenching (CHEQ) effects with Cu^{2+}. With the exception of Cu^{2+}, no significant fluorescence intensity changes were observed in the experiments with the other metal ions. These findings confirm that **1** can be useful as a fluorogenic-sensing material for the selective detection of Cu^{2+} in the presence of other metal ions. This is a rare example of chromogenic sensing of a specific metal ion by functional inorganic nanomaterials. The emission change observed for chemosensor **1** in the presence of Cu^{2+} is reversible by the addition of EDTA. Furthermore, the fluorescein-functionalized silica nanoparticles act as a new type of synthetic fluorogenic chemosensor for imaging Cu^{2+} ions in living cells.

Introduction

Copper is a biochemically essential metal for life; for example, the role of copper–zinc superoxide dismutase in the enzymatic defence against oxygen toxicity.[1] However, the accumulation of copper in the body leads to many serious human afflictions, including neurodegenerative diseases, such as Menkes and Wilson's diseases, Alzheimer's disease, amyotrophic lateral sclerosis, and prion diseases, probably by its involvement in the production of reactive oxygen species.[2] Furthermore, copper has been identified as an environmental pollutant.[3]

In above the connection, much attention has been given to the design of fluorescence probes for the detection of the copper ion as a result of the selective, sensitive, nondestructive, and fast nature of its emission signals.[4] However, most of the sensors developed so far are kinetically slow with limited detection sensitivity below the permissible level of metal toxins, which results in a lack of control in the remote sensing of pollutant species. Despite their limited use so far, the combination of using nanomaterials as solid supports coupled with supramolecular concepts has led to the development of hybrid materials with improved functionalities and offers a promising approach for simple and efficient detection of heavy metal ions for biological, toxicological, and environmental use.[5–7]

It is clear that the receptor-immobilized nanotubes have some important advantages as a solid chemosensor in heterogeneous solid–liquid phases.[8] Firstly, the nanoparticles would be useful as a selective and efficient adsorbent for specific guest molecules in environmental and biological fields because of its larger surface. Secondly, the nanoparticles can easily be isolated from pollutants by simple filtration. Thirdly, the ability to detect and separate specific guest molecules by the functionalized nanoparticles is not dependent on the solubility of the nanoparticles. In particular, the hybrid nanoparticles would be useful as sensing materials in biological systems, as solubility is no longer a factor. Although a few sensing systems for the detection of copper are now known, no precedents exist for the use of hybrid nanoparticles to detect copper ions in living cells. Herein, we report the synthesis of fluorescein-functionalized silica nanoparticles (**1**) and their application for the detection of copper ions in aqueous solution and in living cells.

Results and Discussion

For chemosensor **1**, we chose silica nanoparticles as a supporting material because they are useful for creating

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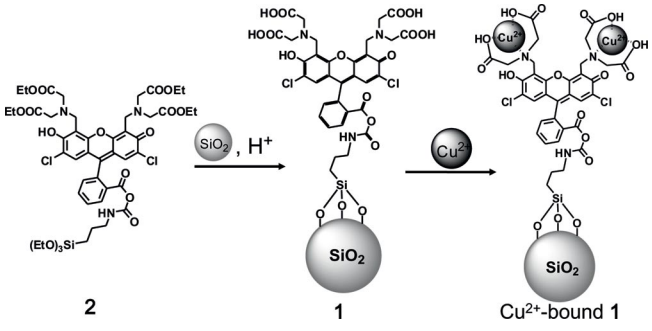
functional hybrid nanomaterials by the grafting method owing to the ease of covalently attaching suitable functional organic molecules and because they are not toxic in vivo.[9] The fluorogenic receptor **2**, adapted with the nanotechnology mentioned above, leads to a new class of chemosensors (**1**), which appears to be an ideal candidate for the detection of copper ions with high efficiency.

Treatment with diethyl iminodiacetate fluorescein[10] and aminopropyltriethoxy silane as precursor for the sol–gel reaction afforded compound **2** as a yellow powder (Scheme 1 and S1). Then, fluorescein-functionalized silica nanoparticle **1** was produced through a grafting reaction, heated under reflux for 24 h in toluene. In this process, the triethoxysilyl group of **2** attached to the silica nanoparticle undergoes hydrolysis and attaches covalently to the surface of the silica nanoparticle.[11–18] The product **1** was characterized by transmission electron microscopy (TEM), UV/Vis and FTIR spectroscopy, and thermogravimetric analysis (TGA) (see Supporting Information).



Scheme 1. Synthetic route of compound **1**.

In Figure 1, the TEM image of **1** clearly shows the silica nanoparticle with diameter of 10 nm. For further structural proof of **1**, we carried out an IR spectroscopy study of both the silica nanoparticle and **1**. For silica, IR peaks appear at 3439, 1630, and 1095 cm^{-1}. For **1** (Figure S1), IR peaks appear at 3382, 2921, 2851, 1645, 1608, 1467, 1190, and 1087 cm^{-1}. These additional peaks originate from the fluorescein-based receptor **2** and provide solid evidence that **2** is indeed attached to the surface of the silica nanoparticles. Furthermore, from the results of the TGA measurement (Figure S2), we determined that **1** consists of only 4.5 wt.-% of **2**.

We then investigated the spectroscopic properties of **1** with the metal ions Cu^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Hg^{2+}, Mg^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+} in aqueous solution (pH = 7.4, all as the perchlorate salt, 100 equiv. with respect to **2** anchored to the silica nanoparticle). The fluorescence spectra were obtained by excitation of the fluorescein fluorophore at 505 nm; both the excitation and emission slits were 1.5 nm. Interestingly, upon addition of Cu^{2+} in H_2O suspension of **1**, **1** displays large chelation-enhanced quenching (CHEQ) effects with Cu^{2+} (Figure 2). In the fluorescence spectrum, free **1** exhibits an emission maximum at 526 nm (Φ = 0.81). Upon addition of increasing Cu^{2+} concentration, λ_{em} is

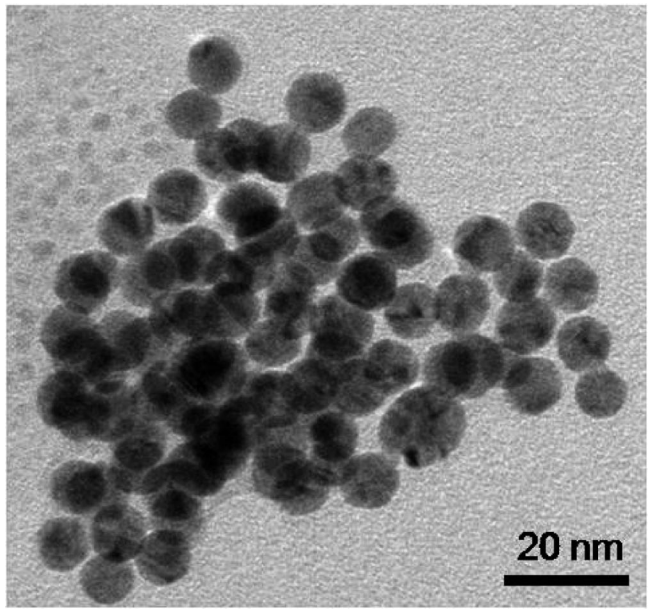


Figure 1. TEM image of rhodamine-functionalized silica nanoparticle **1**.

gradually quenched ($\Phi < 0.0001$, Figures S3 and 2A). With the exception of Cu^{2+}, no significant fluorescence intensity changes were observed in the experiments in which other metal ions were used (Figures 2 and S4). These findings confirm that **1** can be useful as a fluorogenic-sensing material for selective detection of Cu^{2+} in the presence of other metal ions. This is a rare example of chromogenic sensing for a specific metal ion by functional inorganic nanomaterials.

We also probed the binding abilities of **2** for metal ions on the basis of the fluorescence changes upon addition of Cu^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+} in an acetonitrile solution (Figure S5), because **2** is insoluble in water. Unlike **1**, compound **2** shows quenching effects with Hg^{2+}, Cd^{2+}, Zn^{2+}, as well as Cu^{2+}. This difference suggests that chemosensor **1** has the potential to be utilized as a selective fluorescence chemosensor for Cu^{2+}. A highly selective Cu^{2+} recognition/reactivity of fluorescence chemosensor **1** demonstrates that this approach can cooperatively enhance and control the selectivity toward metal ions.

By measuring the emission maximum of Cu^{2+}-bound **1**, the fluorescence change for **1** is found to correspond to the range 0–8 μM of Cu^{2+} (Figure S6). We then found that **1** shows a detection limit of 0.5 μM, which is more than sufficient to sense the Cu^{2+} concentration in drinking water with respect to the U.S. EPA limit (≈20 μM).

In addition, we confirmed the reversibility of the fluorescence change of **1** by removing the Cu^{2+} ion bound to **1** by treatment with EDTA. As expected, on irradiation with UV light, the weak emission signal of **1** in the presence of Cu^{2+} ion returns to a strong green emission signal upon treatment with EDTA (Figure 3). Once again, the emission change is reversible. Because the Cu^{2+} ion bound to **1** is dissociated by EDTA, **1** can be repeatedly used by renewed

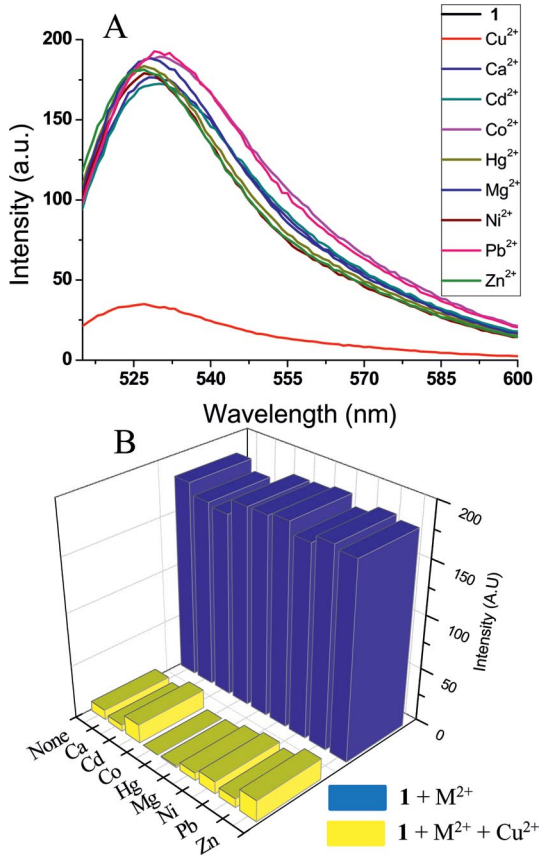


Figure 2. (A) Fluoresence emission changes of **1** (10 μM) upon addition of Cu^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Hg^{2+}, Mg^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+} in aqueous solution (pH = 7.4, all as the perchlorate salt, 100 equiv.). (B) Fluorescence responses of **1** (10 μM) to various metal ions. Blue bars represent the addition of selected metal ions (100 equiv.) to a 10 μM solution of **1**. Yellow bars represent subsequent addition of Cu^{2+} (100 equiv.) to the solution. For all measurements, the pH value was adjusted by using 20 mM HEPES (pH 7.4). Excitation was provided at 505 nm and the emission was monitored at 526 nm.

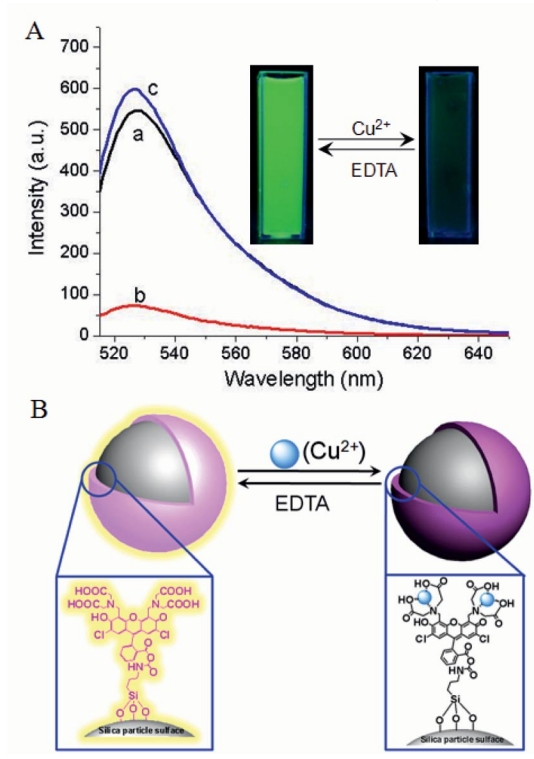


Figure 3. (A) Fluorescence spectra of 10 μM **1** (a) without and (b) with Cu^{2+} (100 equiv.) (c) after treatment with EDTA in water. (B) Proposed structure of Cu^{2+}-bound **1** before and after treatment of EDTA.

treatment with EDTA (Figure 3). After the treatment with EDTA, the fluorescence spectrum of Cu^{2+}-bound **1** is unchanged in the presence of Ca^{2+}, Cd^{2+}, Co^{2+}, Hg^{2+}, Mg^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+} (Figure S7), which indicates that **1** shows great promise as a useful selective chemosensor for the detection of Cu^{2+} in vivo. The fluorescence change was reproducible over several cycles of detection–separation. A Job's plot of the fluorescence changes indicates a 1:2 binding for **1** with Cu^{2+}. From the fluorescence titrations (Figure S3), the association constant (K) for Cu^{2+} coordination to **1** is calculated to be 1.05×10^5 M^{-1} ($\log K = 5.02$).

To determine the cell permeability of **1**, Hela cells (human cancer cells) were incubated with 5.0 μM **1** for 30 min at 37 °C and washed with PBS to remove the remaining **1**. Significant confocal imaging changes of the medium were clearly observed upon addition of 5.0 μM $Cu(ClO_4)_2$ over 30 min at 37 °C. Hela cells incubated with **1** initially display a strong fluorescence image, but the fluorescence image immediately became faint in the presence of Cu^{2+} ion (Figure 4). In addition, **1** was aggregated into vesicles or vacuoles in the cell. These experiments show that **1** is cell-permeable and can respond to changes in intracellular [Cu^{2+}] within living cells. It should thus be potentially useful for the study of the toxicity or bioactivity of Cu^{2+} in living cells.

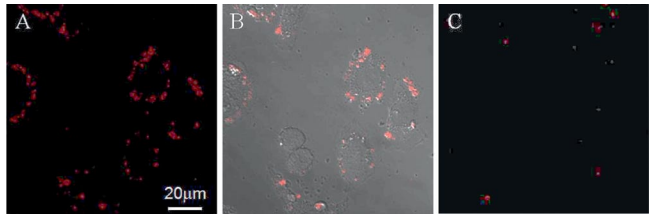


Figure 4. Confocal fluorescence images of live Hela cells. The excitation wavelength is 505 nm and the emission wavelength is526 nm range. (A) Fluorescence image of Hela cells incubated with 5.0 μM **1** for 30min at 37 °C. (B) Bright-field transmission image of (A). (C) Fluorescence image of Hela cells futher incubated with 5.0 μM $Cu(ClO_4)_2$ for 30 min at 37 °C.

To prove the uptake of **1**, we performed a flow cytometry analysis of the cells that had been incubated with **1**. The fluorescence of the cells increases with increasing incubation time (Figure S8). This result shows that the nanoparticles **1** are accumulated in the live cells. In addition, the fluorescence of the cells was quenched with the Cu^{2+} ion, which indicates that the nanoparticle **1** can be used to detect Cu^{2+} ions in intracellular of living cells.

Conclusions

We have readily prepared fluorescein-functionalized silica nanoparticles (**1**), which act as a new type of synthetic fluorogenic chemosensor for imaging Cu^{2+} ions in living cells. Chemosensor **1** exhibits a high affinity and high selectivity for Cu^{2+} over other competing metal ions tested, and can be used to successfully detect Cu^{2+} in cultured cells. These findings show considerable promise for the development of a new category of tailor-made biocompatible systems built by immobilization of appropriate fluorescence receptors on the surface of other novel nanomaterials for fluorescence microscopic imaging as well as for study of the biological function of heavy metal toxins.

Experimental Section

General Methods: Unless otherwise noted, materials were obtained from commercial suppliers and were used without further purification. Flash chromatography was carried out on silica gel 60 (230–400 mesh ASTM; Merck). Thin layer chromatography (TLC) was carried out by using Merck 60 F_{254} plates with a thickness of 0.25 mm. Preparative TLC was performed by using Merck 60 F_{254} plates with a thickness of 1 mm. Transferrin was purchased from Sigma as the iron free form. Melting points were measured with a Büchi 530 melting point apparatus. ^{1}H NMR and ^{13}C NMR spectra were recorded by using Bruker 250 or Varian 500 instruments. Chemical shifts are expressed in ppm and coupling constants (*J*) in Hz. Mass spectra were obtained by using a JMS-HX 110A/110A Tandem Mass Spectrometer (JEOL). UV absorption spectra were obtained on a UVIKON 933 Double Beam UV/Vis Spectrometer. Fluorescence emission spectra were obtained by using a RF-5301/PC Spectrofluorophotometer (Shimadzu).

Photospectroscopy: Fluorescence emission spectra were recorded with a Shimadzu RF-5301-PC instrument. Stock solutions (0.01 M) of the hydrated metal perchlorate salts were prepared in H_2O at pH 7.4. Stock heterogeneous solutions of **1** were prepared in H_2O. For all measurements, excitation was at 505 nm, with excitation and emission slit widths of 1.5 nm. The pH value was adjusted by using 20 mM HEPES (pH 7.4). Fluorescence quantum yields were determined by reference to methylene blue (Φ = 0.04).[10]

Cell Incubation: Hela cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GibcoBRL, USA) supplied with 10% fetal bovine serum (FBS), 50 μg/mL penicillin, and 50 μg/mL streptomycin. The cells were grown on a microscopic culture dish (diameter, 35 mm) with a poly-L-lysine coating. To determine the cell permeability of **1**, the cells were incubated with 5.0 μM **1** for 30 min at 37 °C and washed with phosphate-buffered saline (PBS) to remove the remaining **1**. To observe the fluorescence changes of the medium, 5.0 μM $Cu(ClO_4)_2$ was added into the **1**-loaded cells and then further incubated for 30 min at 37 °C. The reduction of the fluorescence was observed with a confocal microscope. In addition, the Hela cells were incubated with 50 μM **1** for 20 or 30 min. After washing with PBS, the cells were collected and analyzed by flow cytometry (FACSCalibur, BD, USA). Fluorescence was detected on a FL2 channel (excitation: 488 nm, emission: 564–606 nm). A count of 20000 cells were analyzed.

Fluorescence Imaging Experiments: Confocal fluorescence imaging was performed with a Zeiss LSM510 Meta laser scanning microscope and a 40× oil-immersion objective lens, by using the image Pro Plus 5.1 software. Excitation of **1**-loaded cells at 505 nm was carried with a HeNe laser, and emission was acquired at 526 nm. For all imaging on a microscope, the microscopic incubation chamber (Chamlide TC, LCI, Korea) was used at 37 °C in 5% CO_2-humidified air.

3: Compound **3** was prepared by a method reported previously.[9]

2: A solution of **3** (40 mg, 0.094 mmol) and triethylamine (0.03 mL, 0.02 mmol) at 70 °C was treated with 3-(triethoxysilyl)propyl isocyanate (0.054 mL, 0.20 mmol). The reaction mixture was stirred overnight at 70 °C and then cooled to room temperature. After removal of solvent, the product was purified by flash column chromatography on silica gel, by elution with ethyl acetate to provide the title compound **2**(65 mg, 75%). ^{1}H NMR ([D$_6$]acetone): δ = 9.76 (s, 1 H), 8.17 (d, *J* = 13.17 Hz, 1 H), 7.88 (t, *J* = 5.07 Hz, 1 H), 7.34 (m, 2 H), 6.7 (s, 1 H), 6.61 (s, 2 H), 4.4 (m, 10 H), 3.84 (m, 6 H), 3.63 (s, 2 H), 3.2 (s, 8 H), 3.06 (t, *J* = 7.17 Hz, 2 H), 1.59 (m, 2 H), 1.3 (m, 21 H), 0.61 (t, *J* = 3.6 Hz, 2 H) ppm. ^{13}C NMR ([D$_6$]acetone): δ = 172.1, 171.6, 170.6, 157.6, 157.2, 151.2, 147.5, 145.2, 140.0, 139.6, 138.1, 135.6, 135.3, 134.9, 134.5, 125.2, 115.8, 112.4, 111.0, 107.1, 60.7, 59.8, 57.9, 57.7, 54.1, 51.3, 29.3, 17.9, 13.7, 8.6 ppm. MS (FAB) *m/z* = 1050.46 [M + H]$^+$. $C_{30}H_{25}Cl_2N_2O_{13}$ (692.44): calcd. C 51.18, H 4.83, Cl 7.55, N 4.48; found C 51.53, H 4.90, Cl 7.37, N 4.38.

1: Compound **2** (50 mg, 0.054 mmol) was dissolved in anhydrous thf (5 mL) to which the silica nanoparticles (100 mg) were added, and it was stirred under reflux in N_2 for 24 h. The solutions of the functionalized silica nanoparticles were centrifuged for 30 min and redispersed in aqueous solution after the supernatant was removed. The particles were washed three more times and finally redispersed in the detection buffer. IR (on a KBr plate): $\tilde{\nu}$ = 3439.73, 7530.77, 1066.44, 966.91, 796.90, 456.33 cm^{-1}; **1**: 3387.49, 2921.64, 2851.30, 1546.84, 1559.09, 1457.13, 1190.03, 1037.83, 914.78, 797.28, 668.32, 576.41, 461.56 cm^{-1}.

Supporting Information (see footnote on the first page of this article): Additional infrared and fluorescence spectra, thermograms and photographs showing the effects of adding metal ions to **1**.

Acknowledgments

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[1] a) G. K. Walkup, B. Imperiali, *J. Am. Chem. Soc.* **1996**, *118*, 3053–3054; b) K. Venkatesan, F. J. Fernández, *Chem. Commun.* **2003**, 2006; c) M. Montalti, L. Prodi, N. Zaccheroni, *J. Mater. Chem.* **2005**, *15*, 2810–2814.

[2] A. Miyawaki, J. Llopis, R. Helm, J. M. McCaffery, J. A. Adams, M. Ikura, R. Y. Tsien, *Nature* **1997**, *388*, 882–887.

[3] M. M. Henary, C. J. Fahrni, *J. Phys. Chem. A* **2002**, *106*, 5210–5220.

[4] a) Z. Xu, Y. Xiao, X. Qian, J. Cui, D. Cui, *Org. Lett.* **2005**, *7*, 889–892; b) S. H. Kim, J. S. Kim, S. M. Park, S.-K. Chang, *Org. Lett.* **2006**, *8*, 371–374; c) L. Zeng, E. Miller, A. Pralle, E. Y. Isacoff, C. J. Chang, *J. Am. Chem. Soc.* **2006**, *128*, 10–11; d) K. M. K. Swamy, S.-K. Ko, S. K. Kwon, H. N. Lee, C. Mao, J.-M. Kim, K.-H. Lee, J. Kim, I. Shin, J. Yoon, *Chem. Commun.* **2008**, 5915–5917; e) X. Chen, M. J. Jou, H. Lee, S. Kou, J. Lim, S.-W. Nam, S. Park, K.-M. Kim, J. Yoon, *Sens. Actuators B* **2009**, *137*, 597–602; f) H. S. Jung, P. S. Kwon, J. W. Lee,

J. I. Kim, C. S. Hong, J. W. Kim, S. Yan, J. Y. Lee, J. H. Lee, T. Joo, J. S. Kim, *J. Am. Chem. Soc.* **2009**, *131*, 2008–2012.
[5] F. Turner, *Science* **2000**, *290*, 1315–1317.
[6] P. Chen, C. He, *J. Am. Chem. Soc.* **2004**, *126*, 728–729.
[7] T. Gunnlaugsson, J. P. Leonard, N. S. Murray, *Org. Lett.* **2004**, *6*, 1557–1560.
[8] a) F. Hoffmann, M. Cornelius, J. Morell, M. Fröba, *Angew. Chem. Int. Ed.* **2006**, *45*, 3216–3251; b) W. S. Han, H. Y. Lee, S. H. Jung, S. J. Lee, J. H. Jung, *Chem. Soc. Rev.* **2009**, *38*, 1904–1915.
[9] A. Na, X. Bai, S. J. Son, S. B. Lee, H. Ghandehari, *Nano Lett.* **2008**, *8*, 2150–2154.
[10] a) E. J. Jun, J. A. Kim, K. M. K. Swamy, S. Park, J. Yoon, *Tetrahedron Lett.* **2006**, *47*, 1051–1054; b) S.-Y. Chung, S.-W. Nam, J. Lim, S. Park, J. Yoon, *Chem. Commun.* **2009**, 2866–2868.
[11] S. J. Lee, J.-E. Lee, J. Seo, I. Y. Jeong, S. S. Lee, J. H. Jung, *Adv. Funct. Mater.* **2007**, *17*, 3441–3446.
[12] S. J. Lee, S. S. Lee, J. Y. Lee, J. H. Jung, *Chem. Mater.* **2006**, *18*, 4713–4715.
[13] E. Palomares, R. Vilar, A. Green, J. R. Durrant, *Adv. Funct. Mater.* **2004**, *14*, 111–115.
[14] S. O. Obare, R. E. Hollowell, C. J. Murphy, *Langmuir* **2002**, *18*, 10407–10410.
[15] T. Balaji, S. A. El-Safty, H. Matsunaga, T. Hanaoka, F. Mizukami, *Angew. Chem. Int. Ed.* **2006**, *45*, 7202–7208.
[16] E. Palomares, M. V. Martínez-Díaz, T. Torres, E. Coronado, *Adv. Funct. Mater.* **2006**, *16*, 1166–1170.
[17] R. Metivier, I. Leray, B. Lebeau, B. Valeur, *J. Mater. Chem.* **2005**, *15*, 2965–2973.
[18] M. H. Lee, S. J. Lee, J. H. Jung, J. S. Kim, *Tetrahedron* **2007**, *63*, 12087–12092.

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