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Biocompatible and Highly Sensitive Nitric Oxide Sensor Particles Prepared by Layer-by-layer Assembly

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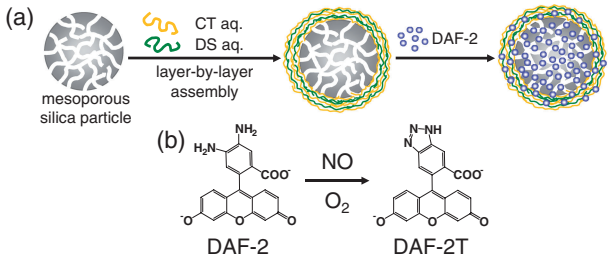
Biocompatible sensor particles with high nitric oxide (NO) sensitivity were fabricated by a combination of layer-by-layer assembly and acid-free chitosan dissolving techniques. The sensor particles encapsulating 4,5-diaminofluorescein (DAF-2), an NO fluorescent probe, can detect 5–500 nM NO and had higher cytocompatibility as compared to DAF-2 molecules. These sensor particles will be useful for quantitative and spatial analyses of extracellular NO molecules from living cells.

Optical fluorescence methods for analyzing intra- and extracellular signaling molecules using fluorescent indicator dyes have been widespread biological tools.[1] Various fluorescent probes have been developed, such as fluorescent seminaphthorhodafluor-1-dye (SNARF-1) for pH changes,[2a] fluo-3 for calcium ion,[2b] RhodZin-3 for zinc ion,[2c] and DAF-2 for nitric oxide (NO),[2d] for detecting cellular events. However, factors such as toxicity to the cell, intracellular sequestration, and protein binding frequently complicate any interpretation of the results.[3] In particular, conventional fluorescent probes have a serious drawback for the quantitative, kinetic, and spatial analyses of the extracellular delivery of signaling molecules from living cells (e.g., the diffusion of NO molecules from vascular endothelial cells to vascular smooth muscle cells). Recently, sensor particles and capsules containing fluorescent probes have been developed to circumvent these problems, and the detection of the local intracellular pH or extracellular NO concentration was reported.[4] However, these sensor particles still have a problem with their cytotoxicity due to the use of non-degradable and non-biocompatible polymers. Furthermore, their sensitivity for targeting signal molecules is not satisfactory for actual cell production.

In the present study, we report for the first time biocompatible NO sensor particles prepared by layer-by-layer (LbL) assembly.[5] NO plays an important role as an inter- and intracellular messenger in the regulation of diverse physiological mechanisms in the cardiovascular system, the central and peripheral nervous systems, and the immune system.[6] Accordingly, a highly sensitive and biocompatible NO sensor will be valuable for biological and biomedical applications. Recently, Zguris et al. reported NO-sensitive hydrogel particles,[4c] but their sensitivity was not enough to detect NO molecules produced from living cells (less than hundreds of nM[6]). Our NO sensor particles composed of an NO fluorescent probe encapsulated in silica particles and biocompatible chitosan (CT)–dextran sulfate (DS) LbL films had both high biocompatibility and NO sensitivity.

The NO sensor particles covered with CT/DS LbL films were prepared by following a procedure based on our previous report on CT/DS hollow capsules[7] (Scheme 1). Silica particles are known as bioinert particles, and CT and DS are biocompatible and biodegradable polysaccharides.[7b] Mesoporous silica



Scheme 1. (a) Schematic fabrication process of the sensor particle. (b) Proposed mechanism in the reaction of DAF-2 with NO in the presence of dioxygen.

particles 1.6 μm in diameter were immersed alternately into a 1 mg mL^{-1} CT solution ($M_w = 6.5 \times 10^5$) containing 25% formic acid (pH 1.6, 0.5 M NaCl) and a 1 mg mL^{-1} DS solution ($M_w = 5.0 \times 10^5$, pH 6.6, 0.5 M NaCl) for 15 min. After repeating the immersion five times, the $(CT/DS)_{2.5}$ films were prepared on the silica particles. The particles were soaked in 5 μM aqueous 4,5-diaminofluorescein (DAF-2) solution for 5 days, and then the DAF-2 encapsulated sensor particles were obtained after washing and subsequent freeze-drying. The encapsulation of DAF-2 was confirmed by measuring the fluorescence spectra with or without a 1-hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC-7; NO donor) solution. The emission spectrum was successfully measured and the maximum fluorescence was exactly the same as the original DAF-2 at 515 nm (Figure 1a). However, intrinsic fluorescence was also measured even without the addition of the NO solution, suggesting the occurrence of some chemical reactions with DAF-2 during the procedure. We assumed that the remaining formic acid molecules contained in the CT/DS films affected the DAF-2 molecules, because Ohmori et al. reported the benzimidazole formation of 1,2-diaminobenzene with formic acid.[8a] In fact, DAF-2 in 25% formic acid solution after adjusting to pH 7.4 showed strong intrinsic fluorescence (Figure S1).[9] Although CT is well known to be soluble in only acidic aqueous solution, we recently reported a novel method for dissolving high-molecular weight CT into water containing 1-hydroxybenzotriazole (HOBt) by the ion complex formation of CT with HOBt.[8b] Accordingly, we prepared the NO sensor particles using a CT–HOBt solution (pH 6.8) by the same procedure mentioned above.[9] The fluorescence spectra of the sensor particles revealed the disappearance of intrinsic fluorescence using the CT–HOBt solution (Figure 1b). Since the intrinsic fluorescence strongly affects the sensitivity, the CT–HOBt solution must be a good candidate for the fabrication of NO sensor particles.

To quantitatively determine the film thickness on the particles, the thickness of the CT–HOBt/DS films was estimated

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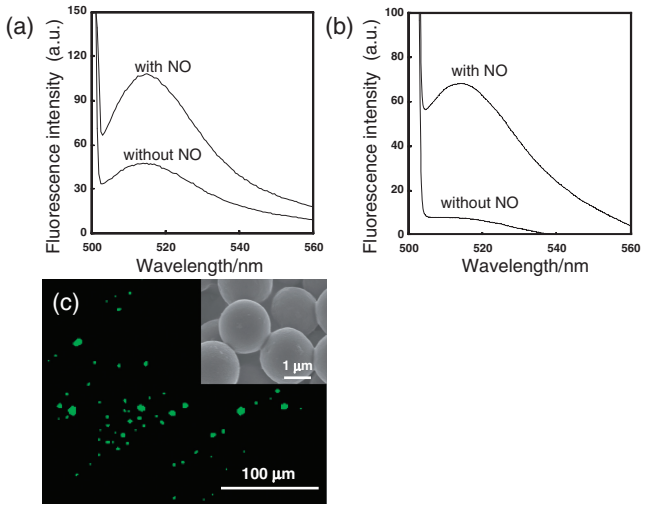

Figure 1. Emission spectra of 1 mg mL^{-1} sensor particles covered with (a) CT/DS and (b) CT–HOBt/DS films in 50 mM Tris-HCl buffer (pH 7.4) with or without 270 nM NO at 37 °C after 15 min of incubation. (c) Fluorescence microscopic image of the sensor particles under the same conditions as (b). The inset is a SEM image of the particles.

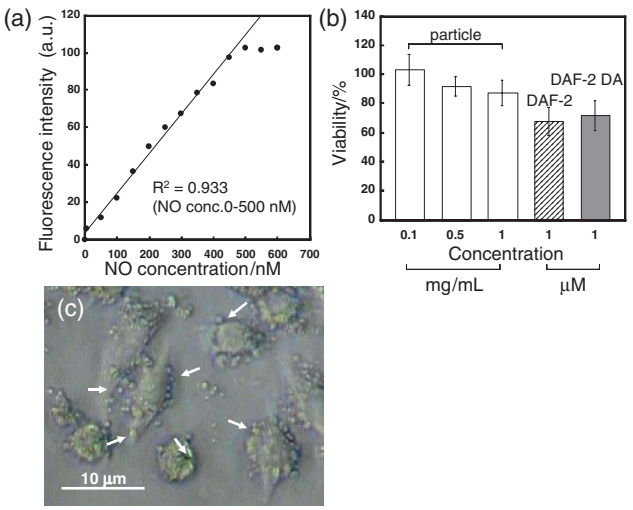

Figure 2. Characterization of the CT–HOBt/DS sensor particles. (a) Fluorescence intensity at 515 nm of 1 mg mL^{-1} sensor particles with varied NO concentration in 50 mM Tris-HCl buffer (pH 7.4) at 37 °C. These intensities were compared to values from the control sample without NO. (b) Fibroblast cell viability with the particles, DAF-2, and DAF-2-DA after 24 h of incubation. The cell number without the samples (control) was defined as 100%. (c) Phase contrast image of the sensor particles on the fibroblast cells. Arrows show the sensor particles.

by a quartz-crystal microbalance (QCM) method (Figure S2).[9] The mean thickness after five-step assemblies was calculated as approximately 40 nm. The DAF-2 would be embedded in the particles by electrostatic interactions with the amine group of CT. Scanning electron microscope (SEM) observations revealed a homogeneous surface of the sensor particles, and fluorescence microscopy observations clearly demonstrated the possibility for in vitro spatial NO imaging (Figure 1c).

The sensitivity of the NO sensor particles was evaluated in NO solutions with varied concentrations (0–650 nM). The NO concentration in the solutions was determined by horseradish peroxidase assay.[8c] The fluorescence intensity in 5–500 nM NO solutions showed a good linear correlation and the intensity at over 500 nM became saturated (Figure 2a). These results clearly demonstrated that the sensor particles can detect several hundred nM of NO molecules (endothelial cell production level), and the detection limit was 5 nM, exactly the same as the original DAF-2 molecule.[2d] The reason for the high sensitivity of these sensor particles seems to be mild fabrication for unstable DAF-2. Average amount of DAF-2 in one particle was estimated to be 3.9×10^{-19} g, and detailed calculation is shown in Supporting Information.[9]

Finally, the biocompatibility of the NO sensor particles was evaluated in relation to DAF-2 and DAF-2 DA (membrane-permeable DAF-2 diacetate), and the concentrations of DAF-2 and DAF-2 DA were set lower than conventionally used levels (10 μM).[6b] Although the sensor particles had high cytocompatibility at over 90%, the cell viability values of DAF-2 and DAF-2 DA were 67 and 71%, respectively (Figure 2b). Furthermore, the fibroblast cells possessing the sensor particles on their cell membrane showed good extended morphology (Figure 2c).

In conclusion, biocompatible NO sensor particles were prepared by LbL assembly and these particles can detect from 5 to 500 nM NO. Quantitative, kinetic, and spatial analyses of the extracellular delivery of NO molecules from vascular endothelial cells are now in progress. These sensor particles, which are stable even after a few months of incubation, may be useful for biological imaging.

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9 Supporting Information is available electronically on the CSJ-Journal Web site, http://www.csj.jp/journals/chem-lett/.

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