科学研究費助成事業　　研究成果報告書

平成　２６　年　　６　月　１７　日現在

機関番号：　１４３０１
研究種目：　若手研究(B)
研究期間：　2012～2013
課題番号：　２４７５０１５９

研究課題名（和文）多孔性金属錯体による時空間制御型一酸化窒素放出プラットフォームの創成

研究課題名（英文）Porous coordination polymers as controllable nitric oxide releasing platforms

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交付決定額（研究期間全体）：（直接経費）　　3,500,000円、（間接経費）　　1,050,000円

研究成果の概要（和文）：一酸化窒素（NO）の細胞内での役割を解明するには、特定の場所に特定のタイミングでNOを正確に放出する材料開発が重要である。本研究では、光応答性多孔性金属錯体を基盤とした新しいNO放出プラットフォームの創成を行った。
光応答性（光刺激によりNOを放出する）の低い分子を多孔性金属錯体として構築することで、NO放出の濃度・タイミングを光の強度・照射時間で制御できた。さらにこの多孔性金属錯体を生体適合性ポリマーに埋め込み、選択的に結晶を光刺激することで局所的なNO放出に成功。放出されたNOが１細胞レベルで取り込まれることを確認した。このプラットフォームにより、NOの様々な生理学的性質の研究が可能となる。

研究成果の概要（英文）：Nitric oxide (NO) stands among the most studied molecules in biomedical sciences because of its numerous physiological properties. This reactive radical is a crucial signaling molecule with site-specific and concentration-dependent activities. Bearing in mind that rapid and precise spatiotemporal control of NO delivery are fundamental criteria that a releasing system should meet, we developed NO-releasing platforms based on photoactive porous coordination polymers (PCPs). Organizing molecules with poor reactivity into PCP structures lead to increased photoreactivity and adjustable NO release via light irradiation. We embedded photoactive PCP crystals in a biocompatible polymer matrix and achieved precisely controlled NO delivery at the cellular level via localized two-photon laser activation. We reported a unique example of spatiotemporally controlled release of NO from PCP-hybrids which can be a valuable tool for further investigations of the physiological role of NO.

研究分野：　化学

科研費の分科・細目：　複合化学・生体関連化学

キーワード：　配位高分子　金属錯体　多孔体　細胞刺激　ナノバイオ　一酸化窒素

１．研究開始当初の背景

Controlled cell stimulation by gaseous bioactive molecules is appealing for investigating cellular mechanisms and signaling networks and for developing new therapeutic approaches. The design of functional scaffolds or devices that can release molecules with precisely controlled timing, dosage and location remains challenging, especially for gaseous molecules, due to handling issues that arise from their high reactivity and physical state. Nitric oxide (NO) is one of the most investigated gasotransmitters, playing a large role in numerous signaling events including proliferation and vasodilatation. Moreover, microenvironmental modulation by endogenous NO is believed to affect both physiological conditions such as synaptic transmission and tissues or cancer stem cells. Great effort has recently been expended on the design of controllable NO-releasing scaffolds; however, despite its value to further investigate a cryptic role of NO, the precise localization of NO delivery at the cellular level has not yet been demonstrated.

Stable compounds that are able to produce NO through a photochemical reaction are promising for achieving temporal control over NO release, and a variety of NO photodonors have recently been developed. Light provides a non-invasive trigger, which can be highly controlled in terms of intensity, wavelength or duration without affecting any important physiological parameters. In addition, photons can be easily manipulated and focused to achieve precisely localized stimulation. To take full advantage of this spatial property, a concentration of photoresponsive molecules at defined locations is necessary for the consistent release of the target compound. Only a few examples consisting in the functionalization of nanoparticles surface or the entrapment of photodonors into porous, non-functional scaffolds such as mesoporous silica have been reported.

２．研究の目的

Aside from other macromolecular scaffolds, porous coordination polymers (PCPs) represent a distinct three-dimensional framework, assembled from metal ions or clusters and functional organic ligands as building units. Designing appropriate NO photodonor ligands would advantageously render the framework itself photoactive, while the loading of NO-donors into pores is no longer essential, and potentially toxic photoadducts remain chemically bound to the framework. The ligands are thereby concentrated in a restricted space that exhibits high light-harvesting and NO-reservoir capacities. We propose a new approach for developing on-demand NO delivery platforms based on photoactive PCPs. The assembly of nitro-containing imidazolate ligands, which have low photoreactivity, into well-defined crystalline coordination frameworks leads to a drastic increase in photoreactivity, and the resulting materials can efficiently release NO, only upon light irradiation. The applicability of these frameworks for localized cellular delivery is investigated by developing hybrid substrates in which microcrystals of photoactive PCPs are embedded into a biocompatible and gas-permeable polymer matrix to allow cellular adhesion. Two-photon laser scanning confocal microscopy experiments allows for chemical modulation of the cellular microenvironment through the spatiotemporally controlled release of NO. The biological relevance of the delivered NO is demonstrated by a transient increase in the intracellular calcium ion concentration in locally stimulated cells following activation of the NO-sensitive membrane protein.

３．研究の方法

Synthesis and characterization of nitric oxide frameworks.

We selected imidazole-based ligands, 2-nitroimidazole (2nIm) and 5-methyl-4-nitroimidazole (mnIm), which are suitable for constructing robust and versatile zeolitic imidazolate frameworks (ZIFs). The solvothermal reaction of zinc nitrate with 2nIm or mnIm in DMF yielded single crystals of [Zn(2nIm)2]n (NOF-1) or [Zn(mnIm)2]n (NOF-2), respectively (Fig. 1) (NOF = nitric oxide framework). Alternatively, homogeneous microcrystalline powders of NOF-1 and NOF-2 were synthesized in a water/DMF mixture at room temperature in the presence of a sodium formate modulator, which induced faster nucleation by ligand deprotonation.

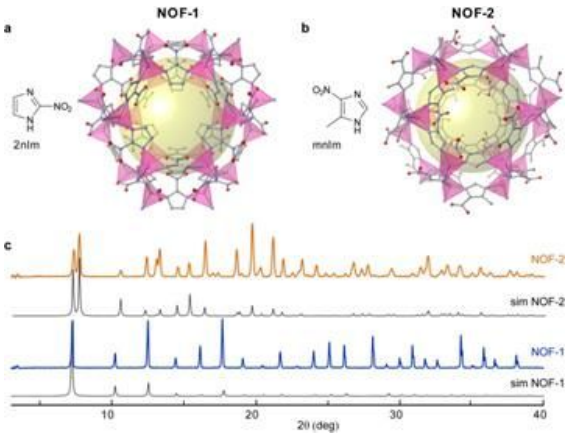

Figure 1. Representation of the nitric oxide-releasing frameworks (a, b) and PXRD diffraction peaks of the NOF-1 and NOF-2 microcrystalline powders (c).

Light-induced nitric oxide release.
The NO-releasing properties of NOF-1 and NOF-2 under light irradiation were probed using the NO selective ozone chemiluminescence technique (Fig. 2a). First, the 2nIm and mnIm ligands presented low photoreactivity and produced only limited quantities of NO after 3 h of sustained illumination (release yield = 1.4% and 5.9% for 2nIm and mnIm respectively, based on the conversion of nitro groups to nitric oxide). However, when these ligands were organized into crystalline frameworks, their photoreactivity exhibited an increase greater than an order of magnitude under the same illumination conditions; NOF-1 and NOF-2 released 3.4 μmol·mg-1 and 2.9 μmol·mg-1 of nitric oxide, corresponding to a conversion yield of 50% and 46%, respectively.

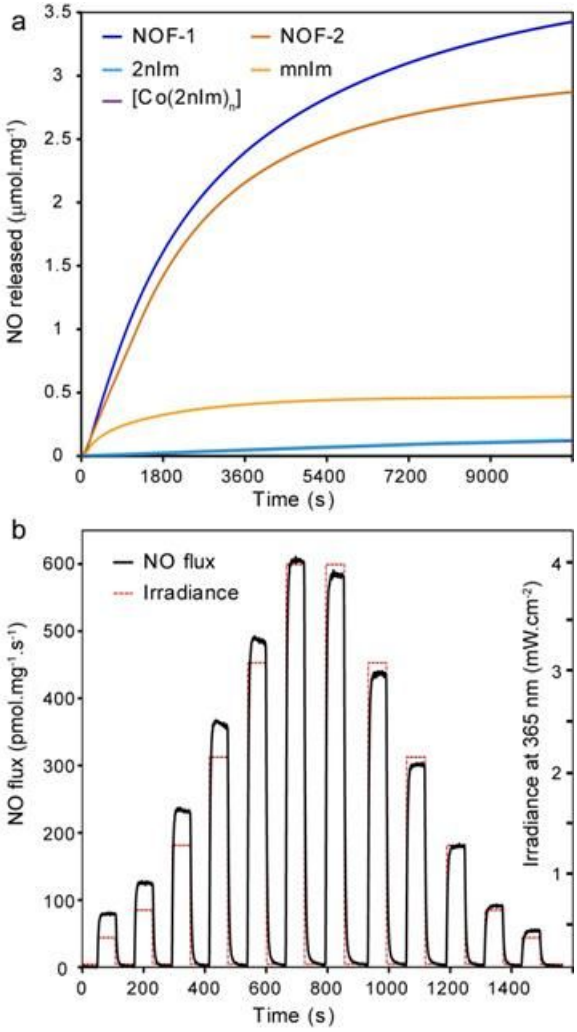

Figure 2. Photo-induced nitric oxide-releasing properties. a) The photoreactivity of the ligands 2nIm and mnIm was drastically enhanced upon the formation of the porous coordination polymers NOF-1 and NOF-2. b) The NO flux produced upon the photoactivation of NOF-1 can be tuned by varying the intensity of the light source. When the irradiation is interrupted, the NO production is instantly terminated, and no leakage is observed in the dark state.

The lack of nitric oxide production by the methylimidazolate-based reference material, [Zn(MeIm)2]n (ZIF-8), supports the photochemical involvement of the nitro groups in the formation of NO. As confirmed by GCMS analysis, NO was the only nitrogen oxide species released after the irradiation of NOF-1.

In addition to high releasing efficiency, spatiotemporal control over the produced NO flux is important to NO delivery systems.

We investigated the illumination of NOF-1 under variable light power (Fig. 2b). Nitric oxide is released instantly upon photoactivation, and the NO flux can be easily tuned by both the irradiation intensity and the amount of irradiated material. As anticipated, the production of NO is immediately interrupted when the irradiation ceases and its concentration quickly drops to trace levels. NOF powders did not present any significant decrease in crystallinity or in NO release after 8 months of storage under ambient conditions. Additionally, the lack of significant NO release when heated up to 200°C, confirmed the thermal stability of NOF powders and that NO is produced exclusively through a photochemical process.

Spatiotemporally controlled NO-release in cellular media.

To render our compounds biologically applicable, we prepared NOF-based substrates suitable for cell cultures and microscopic imaging (Fig. 3a); NOF-1 microcrystals were first spin-cast on a glass-bottomed dish. Next, a biocompatible polydimethylsiloxane (PDMS) layer was spin-coated on top to embed the crystals completely in a polymer matrix with a reproducible thicknes. Notably, the crystal distribution on the glass surface did not seem to be altered by the spin coating process and NOF crystals remained on the bottom part of the polymer layer (Fig. 3b). To deliver NO in a localized cellular environment, we utilized the high spatiotemporal resolution of confocal laser scanning microscopy, which allowed for precise and localized photoactivation of the NOF-1/PDMS substrates and subsequent rapid observation of the cellular response.

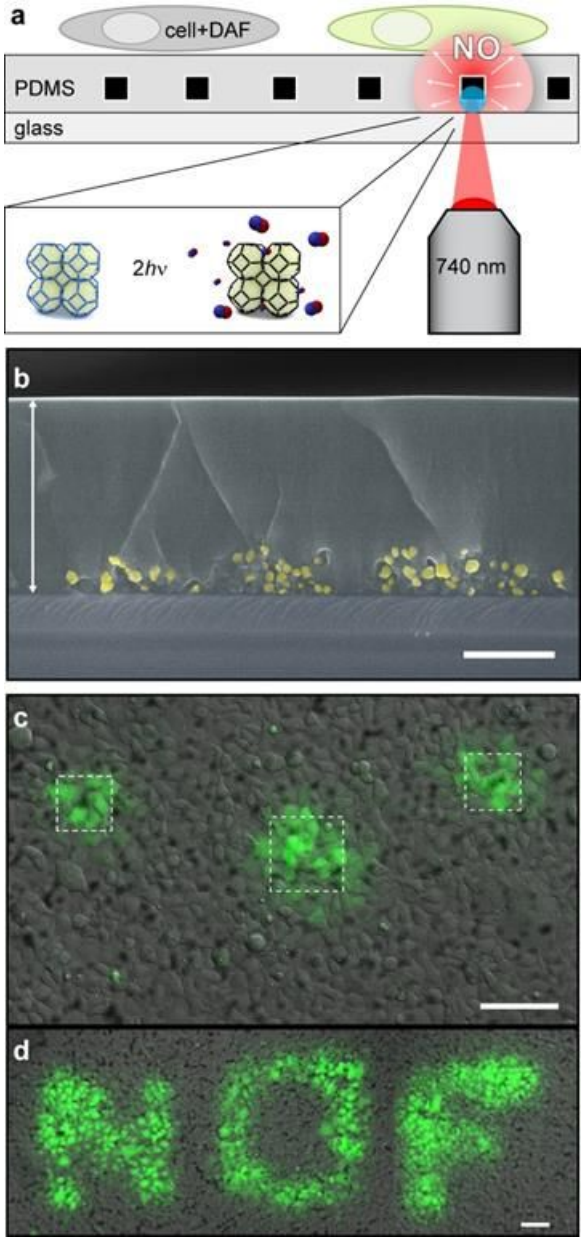

Figure 4. a) Schematic illustration of the localized cell-stimulation platform. b) Cross-section SEM image of NOF-1/PDMS substrate. (scale bar: 10 µm). The colors are superimposed by post-treatment for the sake of clarity. c) Confocal microscopy images of NOF-1-embedded substrates cultured with HEK293 cells introduced via DAF-FM. The selective photoactivation of the NOF-1 crystals (within the areas defined by the white squares) induced a fluorescent response in the surrounding cells, highlighting the localized NO delivery and uptake. (scale bar: 100 µm). d) Further demonstration of spatiotemporal control by writing "NOF" upon activation of the selected regions (scale bar: 100 µm).

Because the absorption of our material was essentially in the UV region, we used a near-infrared two-photon laser to photoactivate NOF-1, thus greatly reducing the risk of photodamaging the cells with harmful UV irradiation while effectively inducing the NO release. The sterilized NOF-1/PDMS were coated with Matrigel™ to facilitate cell adhesion and growth, and then human embryonic kidney 293 (HEK293) cells were cultured on this substrate. Compared to pristine PDMS substrates, the presence of NOF-1 in the polymer layer did not induce any notable cytotoxicity.

We predicted that the high spatial resolution of the two-photon confocal laser would allow for the selective activation of any crystals chosen to enable localized NO delivery to the surrounding cells. Prior to the activation experiments, DAF-FM DA (4-amino-5-methylamino-2′,7′-difluorofluorscein diacetate), a cell-permeable NO fluorescent indicator, was introduced into the HEK293 cells. As shown in Fig. 3c and Fig 3d, the rapid photoactivation of a selected region containing NOF-1 crystals quickly induced an increase in the fluorescence of the surrounding HEK293 cells. Within seconds, the NO generated from NOF-1 was able to diffuse through the PDMS layer to the cellular membrane and further reacted with the NO indicator to form a fluorescent adduct. These results clearly suggest a high degree of spatiotemporal control over the NO delivery using a simple, reproducible and extremely fast two-photon photoactivation process.

４．研究の成果

The spatial segregation of the functional components in porous frameworks, with electronically non-involved metal centers, allowed a drastic increase in the photo reactivity by preventing the quenching of the reactive excited states. This advantageous accumulation of photoactive donors in PCP architectures lead to efficient and tunable light-induced NO release and places NOF materials (3.4 μmol·mg-1 for NOF-1) above the most efficient, controllable solid-state NO-delivery scaffolds (1.3 μmol·mg-1 for nitrosothiol-modified silica xerogels). Notably their excellent stability under ambient conditions avoids the need of cautious handling or storage (as required with light-sensitive metal-nitrosyls or thermally unstable nitrosothiols). This work suggests a great potential of PCPs for controllable light-induced release of biologically relevant molecules.

To take advantage of the concentration of photodonor ligands in the PCP structure, we prepared a new NO cell-stimulation platform by immobilizing the NOF-1 microcrystals in a permeable and biocompatible polymer layer and we confirmed that the irradiation of NOF-1/PDMS could generate relevant and sustained flux of gaseous NO, which easily diffused through the polymer layer. A high degree of spatiotemporal control over the NO delivery was achieved using harmless near-infrared two-photon laser activation and allowed for a precisely localized stimulation of cells surrounding the photoactivated crystals. We have further demonstrated the biological relevance of this exogenous NO delivery by observing the subsequent cellular response, i.e., a transient increase in the intracellular calcium concentration, triggered by the conformational change in a NO-sensitive transmembrane channel. Aside from the potential therapeutic applications, we believe this approach could provide valuable fundamental understanding of the physiological and pathophysiological roles of NO, particularly the functions of NO in brain circuitry in which the spatiotemporal generation of stimulating molecules in subcellular domains is desired.

５．主な発表論文等

（研究代表者、研究分担者及び連携研究者には下線）

〔雑誌論文〕（計　1 件）

① Stéphane Diring, Dan Ohtan Wang, Chiwon Kim, Mio Kondo, Yong Chen, Susumu Kitagawa, Ken-ichiro Kamei, Shuhei Furukawa: “Localized cell stimulation by nitric oxide using a photoactive porous coordination polymer platform”,Nature Commun.2013,4,2684(査読有)
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〔学会発表〕（計　4 件）

① Diring Stephane: “Localized cell stimulation by Nitric Oxide Using Porous Coordination Polymers”錯体化学会第63回討論会,琉球大学(沖縄)（2013年11月2日)(招待講演),

② Diring Stephane: “Spatiotemporally controlled release of Nitric Oxide from Porous Coordination Polimers” Interantional Conference on Photochemistry,KU Leuven(Leuven)(2013年7月23日)

③ Diring Stephane “Porous Coordination Polymers as Spatiotemporally Controllable Nitric Oxide Releasing Platforms” 3rd International Conference on Metal-Organic Frameworks and Open Framework Compounds, John McIntyre Conference Centre (Edimbourg) (2012年9月17日)

６．研究組織

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