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Akzeptierter Artikel

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Zitierweise: Angew. Chem. Int. Ed. 10.1002/anie.202007039

Link zur VoR: https://doi.org/10.1002/anie.202007039

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Monochromophore-Based Phosphorescence and Fluorescence from Pure Organic Assemblies for Ratiometric Hypoxia Detection

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Abstract: Hypoxia is a parameter related to many diseases. Ratiometric hypoxia probes often rely on a combination of an O2insensitive fluorophore and an O2-sensitive phosphor in a polymer matrix, which are comprised by high cost and multi-step synthesis of transition metal complexes. The two-chromophore hypoxia probes encounter unfavorable energy transfer processes and different stabilities of the chromophores. Reported herein is a pure organic ratiometric hypoxia nanoprobe, assembled by a monochromophore, naphthalimide ureidopyrimidinone (BrNpA-UPy) bridged by a bis-UPy functionalized benzyl skeleton. The joint factors of quadruple hydrogen bonding, the rigid backbone of UPy, and bromine substitution of naphthalimide derivative facilitate briaht phosphorescence (Φ_P = 7.7%, τ_P = 3.2 ms) and fluorescence of the resultant nanoparticles (SNPs) at room temperature, which enable the accurate ratiometric sensitive oxygen detection (K_{sv}= 189.6 kPa⁻ ¹) in aqueous solution as well as in living HeLa cells.

Quantification of molecular oxygen (O₂) is of great significance in complex environments and biological fields, e.g. the lack of oxygen named as hypoxia is connected with many diseases. Intracellular O₂ levels or the degree of hypoxia provides an important parameter in the early-stage diagnosis of cancers, cardiovascular diseases, and stroke.[1] Among currently available methods (i.e., positron emission tomography (PET)^[2], magnetic resonance imaging (MRI)^[3], optical detection), ratiometric readout of optical signals at two wavelengths is highly preferable for imaging in vivo O₂ levels due to its simple operation, minimal invasiveness and accuracy [4-8]. To date, most reported ratiometric oxygen sensors rely on a combination of the O2-insensitive fluorophore and O2-sensitive phosphorescent transition metal complex (e.g. Pt(II) porphyrin) in a polymer matrix. However, undesirable energy transfer and different stabilities of the fluorophore and metal complex phosphor in one probe might also compete with readout of oxygen levels. Meanwhile, transition metal complexes would face multi-step synthesis, high cost and unclear toxicity.

A single organic chromophore to generate phosphorescence and fluorescence simultaneously for

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ratiometric oxygen detection, would be promising to overcome the above disadvantages. Particularly, the long-lived character organic phosphorescence will ensure the of efficient phosphorescence quenching by molecular oxygen, thus leading to the sensitive O₂ detection. However, most pure organic compounds could only emit fluorescence at room temperature owing to the spin prohibition of the underlying triplet-singlet emission. [9,10] Recently, delicate strategies such as crystal formation,^[11] host-guest interaction,^[12] polymerization ^[13], as well as halogen and hydrogen bonding^[14] have been employed to increase the occurrence of triplet excited states and decrease their nonradiative decay to promote room temperature phosphorescence (RTP) at solid state. Unfortunately, these strategies are excluded to be a hypoxia sensor owing to their poor water solubility/dispersibility to penetrate into biological systems.



Scheme 1. Phosphorescence and fluorescence enabled by an organic monochromophore in supramolecular polymer assembled nanoparticles (SNPs) for ratiometric oxygen detection. Molecular structures of organic chromophore (BrNpA-UPy), skeleton molecule (Ph-bisUPy), and UPy.

At present, the accurate ratiometric readout of oxygen levels using pure organic monochromphore platform remains largely unexplored. Even for RTP from the self-assembled organic molecules in aqueous solution, they failed to act as hypoxia sensor due to the poor oxygen permeability in the tightly-packed structures.^[15] Impressively, Fraser and coworkers discovered that nanoparticles made from difluoroboron dibenzoylmethane poly(lactic acid) could emit both fluorescence and phosphorescence for ratiometric hypoxia imaging.^[13b] Kim and coworkers presented an oxygen detection platform in aqueous solution and gas phase by a core-shell nanoparticles with an organic chromophore cross-linked polystyrene as core.^[13c] In these carefully chosen polymer matrixes, the longlived organic phosphorescence in water as well as oxygenpermeabilities have been successfully achieved. Inspired by these works, we wondered whether one could use supramolecular polymers of small organic molecules to execute

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hypoxia detection. Representative 2-ureido-4-[1H] pyrimidinone (UPy) moiety as quadruple hydrogen-bonded unit has been established forming supramolecular polymers (a binding constant of up to 6×10^7 M⁻¹ in chloroform).^[16] The facile structural and functional tunability, stimuli responsiveness and good oxygen permeability of these supramolecular polymers^[8a-b] promoted us to explore a small organic molecule based quadruple hydrogen-bonded assembly for ratiometric O₂ detection.

Herein UPy functionalized bromine-substituted а naphthalimide derivative (BrNpA-UPy) as the organic chromophore, and a bis-UPy functionalized benzyl (Ph-bisUPy) as the skeleton molecule are designed to fabricate organic supramolecular polymer assembled nanoparticles (SNPs) (Scheme 1). Intriguingly, BrNpA-UPy emit only fluorescence in organic solvent at room temperature, while the water-dispersible SNPs made of BrNpA-UPy can show phosphorescence and fluorescence simultaneously at room temperature. The synergistic interaction of the quadruple hydrogen bonds to form backbone and the heavy atom effect of bromine are believed responsible for the observed RTP and fluorescence from BrNpA-UPy (see below). Most importantly, these SNPs could ratiometrically and quantitatively detect hypoxia (K_{sv}=189.6 kPa⁻¹) in water and in living cells. To our best knowledge, this is the first example to realize ratiometric hypoxia sensing based on supramolecular assemblies of small organic monochromophore.



Figure 1. (a) SEM and TEM (inset) of SNPs (Ph-bisUPy: BrNpA-UPy = 5:1). (b) Steady-state emission spectra of SNPs-dispersed aqueous solution under air and nitrogen (blue: Air; red: N₂; λ_{ex} = 348 nm); Inset: Luminescence photographs of SNPs-dispersed aqueous solution under 365 nm UV irradiation under air and nitrogen.

Ph-bisUPy and BrNpA-UPy were facilely synthesized from cheap starting materials. [17-18] SNPs from certain ratios of PhbisUPy and BrNpA-UPy in aqueous solution were prepared by miniemulsion method (see Supporting Information for details). Driven by the guadruple hydrogen bonding in chloroform, the first step is the dimerization of UPy moieties, then the emulsified organic droplets in water enabled aggregation to form SNPs shown in Scheme 1 and Figure S1. [17a, 19] Morphological characterization by scanning electron microscopy (SEM) and transmission electron microscope (TEM) indicated the formation of stable water-dispersible SNPs with a particle size of ca. 240 nm (Figures 1a and S2). The absorption spectra of the SNPs exhibited the strong characteristic absorption peak at 275 nm, and 348 nm, 365 nm for Ph-bisUPy and BrNpA-UPy, respectively, indicating the successful co-assembly of chromophore with the skeleton molecules (Figure S3a and 4a). As shown in Figure 1b, upon excitation at 348 nm, SNPsdispersed aqueous solution exhibited two distinct emission

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bands at 396 nm and 570 nm under nitrogen, with bright orangered emission color observed. The redshift of 174 nm between these two emission peaks, the nanosecond and microsecond lifetimes of the two emission bands suggest the assignment of the higher energy band at 396 nm as fluorescence and stronger lower energy band at 570 nm as phosphorescence. Furthermore, the emission band at 570 nm totally disappeared under air, further indicating its phosphorescence character and oxygensensitivity. Together with the total transparency of Ph-bisUPy at the excitation wavelength (Figure S3), we believe that a pure organic assembled nanoparticle with intense phosphorescence and fluorescence from BrNpA-UPy in aqueous solution at room temperature have been successfully prepared.

Next, SNPs with different ratios of BrNpA-UPy and PhbisUPy were investigated to identify and optimize the optimal ratios of BrNpA-UPy on the phosphorescence properties. As shown in Figures 2a and S5a, the phosphorescence quantum yield reached the maximum as 7.7% at the ratio of Ph-bisUPy: BrNpA-UPy = 5:1 under nitrogen, which is the most efficient pure organic RTP in aqueous solution reported so far. The phosphorescence lifetime also reached the maximum as 3.2 ms at ratio of 5:1 under nitrogen (Figure S5b), suggesting the minimal self-quenching and the rigid molecular environment of SNPs at this ratio. As noted above, the efficient long-lived phosphorescence from SNPs in aqueous solution is in contrast to negligible RTP observed for BrNpA-UPy in CHCl₃ (Figure S3b).

To further understand the phosphorescence enhancement of SNPs in aqueous solution, we studied the contribution of the UPy backbone, heavy atom effect and guadruple hydrogen bonding. Firstly, control nanoparticles prepared from BrNpA-UPy (SNP-1) in the absence of Ph-bisUPy by the same miniemulsion method exhibited a cubic mass (Figure S6a). Under the same excitation conditions, similar phosphorescence peaks at 570 nm but with lower intensity and shorter lifetime ($\tau_p = 0.2$ ms) than those of SNPs were observed in SNP-1 (Figures 2c, S6 and Table S1). Obviously, the skeleton molecule Ph-bisUPy plays an important role in the formation of well-dispersed SNPs for efficient phosphorescence. Secondly, control nanoparticles SNP-2 from Ph-bisUPy and a bromine-free compound (NpA-UPy) were prepared. Upon the introduction of halogen, spin-orbit coupling between singlet excited state and triplet excited state can facilitate the intersystem crossing. [13b, 20] Thus, SNP-2 exhibited much lower phosphorescence quantum yield (Φ_p =1.2%) than those of SNPs (Figures 2 and S7). At last, control nanoparticles from Ph-bisUPy doping with phosphor molecule (BrNpA) in the absence of UPy units (SNP-3, Ph-bisUPy: BrNpA=5:1) showed decreased phosphorescence quantum yield and lifetime to 3.9% and 2.5 ms as compared with SNPs under identical conditions, Figures 2 and S8. The differences of these nanoparticles are also evident by their emission colors pictures and the Commission Internationale de L'Eclairage (CIE) 1931 chromaticity diagram (Figure S9). [21] All of the observations suggest that synergistic interaction of heavy atom effect and quadruple hydrogen bonding promote the production of triplet excited state and suppress the vibrational and rotational nonradiative decay of organic molecules, thus remarkably enhancing the phosphorescence and fluorescence from SNPs in aqueous solution (Figure S10).

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Figure 2. (a) Phosphorescence quantum yields (red) and lifetimes (blue) at different molar ratios of SNPs. (b) The control nanoparticles SNP-1 from BrNpA-UPy, SNP-2 from NpA-UPy and Ph-bisUPy (5:1), SNP-3 from BrNpA and Ph-bisUPy (5:1). (c) Steady-state phosphorescence spectra of SNPs (red line), SNP-1 (green line), SNP-2 (blue line), and SNP-3 (pink line) in aqueous solution under N₂ (λ_{ex} =348 nm, delay: 0.1 ms). (d) Phosphorescence quantum yields and lifetimes of SNPs, SNP-2 and SNP-3 in aqueous solutions under nitrogen.

Subsequently, the dually emissive SNPs at the ratio of PhbisUPy: BrNpA-UPy = 5:1 in aqueous solutions was employed for the ratiometric O₂ sensing experiment for its optimized fluorescence/phosphorescence intensity ratio and long lifetime. As shown in Figure 3a, upon excitation, the phosphorescence of SNPs at 570 nm is gradually quenched by introduction of different levels of oxygen, and the fluorescence at 396 nm is hardly affected by changes in oxygen concentration, which functions as a perfect internal reference signal for ratiometric analysis. To quantify the oxygen sensitivity of SNPs, R is defined as the ratio of phosphorescence intensity (I_P) at 570 nm to that of the fluorescence intensity (I_F) at 396 nm (R = I_P/I_F), the dependence of the intensity ratio R_{N2}/R as a function of oxygen partial pressure are analyzed according to Stern-Volmer equation.

$$\frac{R_{N2}}{R} = 1 + K_{SV}[0_2] \tag{1}$$

To our delight, the optimal linear range of the oxygen nanoprobe was found at the oxygen levels of 0-3% (Correlation coefficient $R^2 = 0.994$, Figure 3b), which are in good correspondence with biological hypoxia contexts.^[22] Sensitive ratiometric oxygen detection is closely related to the bright phosphorescence, long decay lifetime and the oxygen permeability of the material. [4a] As a result, the oxygen quenching efficiency (Q) is calculated to be 95.5% (See Supporting Information for details). The Stern-Volmer constant (K_{SV}), which is another important index to indicate the sensing ability of SNPs for oxygen, was determined to be 189.6 kPa⁻¹. This is a significant improvement over the Pt(II) porphyrin based bichromophoric ratiometric oxygen nanosensor (K_{SV} = 23.6 kPa⁻ 1).[8a-b] What's more, the R value, independent on the probe concentration, remained practically unaltered at a determined oxygen level (Figure S11a). On the other hand, the phosphorescence lifetimes of SNPs at 570 nm decreased dramatically with increasing oxygen levels (3.2 ms, 1.2 ms and 94 μ s for N₂, 1% O₂ and 21% O₂. Figure S12). These are consistent with the collisional quenching of phosphorescence by oxygen. [8] As expected, the intensities of the fluorescence and phosphorescence decreased at higher temperature (Figure S11). ^[23] Similar trends as SNPs at ratio of 5:1, the K_{SV} was obtained as 90.9 kPa⁻¹ and 81.2 kPa⁻¹ for SNPs at ratios of 10:1 and 30:1, respectively (Figure S13-14). Furthermore, the electron spin resonance (ESR) signal corresponding to singlet oxygen adduct is significantly enhanced upon illuminating mixture of SNPs and 2, 2, 6, 6-tetramethylpiperidine (TEMP) (Figure S15), confirming the production of singlet oxygen through the triplet-triplet energy transfer process.



Figure 3. (a) Ratiometric spectral responses of the SNPs-dispersed aqueous solution at varied oxygen levels (0%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%, 0.6%, 0.8%, 1.0%, 1.5%, 2.0%, 3.0%, 4.0%, 8.0%, 14.0%, 21.0%, 100%). (b) Linear Stern–Volmer plot of R_N/R as a function of oxygen level. Inset: at large oxygen levels shown for comparison: more significant curvature approaching ambient level (21%) (c) Steady-state emission spectra of SNPs in aqueous solution under nitrogen before and after storage for 3 months at room temperature in the dark. (d) Reversible oxygen detection of SNPs under oxygen-free/oxygen-saturated cycles. (λ_{ex} = 348 nm, c=2.1×10⁻⁵ M)

Stabilities are very important factor of an excellent nanoprobe. As indicated by the measured steady-state luminescence spectra of SNPs, no obvious spectral changes were observed after over 3 months' storage at room temperature, indicating its excellent storage stabilities (Figure 3c). Moreover, all signal changes are fully reversible under oxygen-free and oxygen-saturated cycle (Figure 3d). The photostabilities of the SNPs were also examined under irradiation by a 365 nm LED lamp (19 mW·cm⁻²). Even after 3 hours of exposure, UV-vis absorption spectra hardly showed significant changes (Figure S16). The above results indicate this organic monochromophore based optical ratiometric oxygen nanoprobe possesses very high oxygen sensitivity, excellent storage stability, optical stability and cycle stability. When SNPs were applied in the with N-ethylmaleimide microenvironments (NEM), Nacytelcysteine (NAC), and glutathione (GSH) for biomedical detection, the phosphorescence intensities were independent of these small biomolecules (Figure S17), implying our hypoxia nanoprobe was capable of measuring O₂ levels in the intracellular environment without interference.

Given the advantages of the organic nanoprobe, we applied it for hypoxia detection and imaging in living cells. Here HeLa cell was chosen as a biometric model. The cytotoxicity of the nanoprobe was excellent as demonstrated by a standard CCK-8 cell active reagent assay (Figure S18), which facilitate the biological application of SNPs. Then, the performance of SNPs

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as ratiometric sensor in hypoxia imaging in HeLa cells was investigated using confocal laser scanning microscopy (CLSM, Figure 4). When excited by 405 nm laser, blue and red luminescence observed in the cytoplasm of the cells indicated that the SNPs can easily penetrate the cell membrane and enter into the cytoplasm. The blue (425-475 nm, Figure 4b, 4f) and the red emission channels (570-620 nm, Figure 4c, 4g) are assigned to the fluorescence and phosphorescence of the organic chromophore BrNpA-UPy in SNPs, respectively. At 0% and 21% of O₂ atmosphere, the fluorescence intensity was nearly identical, while the phosphorescence intensity was significantly enhanced at 0% O₂ than 21% O₂. The significant change in the I_{red} / I_{blue} ratio in living cells indicates that the SNPs sensor can be intracellularly hypoxic inductive (Figure 4d, 4h).



Figure 4. Confocal luminescence images of HeLa cells incubated with SNPs (1.1× 10⁻⁶ M in water) at 21% O₂ (a-d) and 0% O₂ (e-h) partial pressure. (a, e) Images of bright-field; (b, f) Images of blue channel (425-475 nm); (c, g) Images of red channel (570-620 nm); (d, h) Images of red/blue ratio. In ratiometric imaging, the ratio of emission intensity at 570-620 nm to that at 425-475 nm (I_{red} / I_{blue}) was chosen as the detected signals (λ_{ex} = 405 nm). Scale bar: 10 µm

In summary, we have developed an organic monochromophore based ratiometric hypoxia nanoprobe from quadruple-hydrogen bonded assembled nanoparticles. These nanoparticles can emit efficient long-lived phosphorescence (Φ_P = 7.7%, T_P = 3.2 ms) and fluorescence simultaneously from BrNpA-UPy, which could function as oxygen indicator and internal standard, respectively. Dual emission from a single chromophore is obviously advantageous over bischromophoric ratiometric sensor models with better accuracy, homogeneity, reproducibility and stability. The important role of quadruple hydrogen bonding, the rigid backbone, as well as the bromine substitution facilitate bright phosphorescence in aqueous solution. More importantly, these UPy-based organic SNPs possess excellent oxygen permeability to accurately detect hypoxia ratiometrically in aqueous solution and in living HeLa cells as well. We believe this unique pure organic hypoxia sensor would be of great potential in biomedical and biotechnological applications, which is actively undergoing in our laboratory.

Acknowledgements

Financial support from the National Natural Science Foundation of China (Grants 21871280 and 21861132004), the Ministry of Science and Technology of China (Grant 2017YFA0206903), the Strategic Priority Research Program of the Chinese Academy of Sciences (Grant XDB17000000), the Key Research Program of Frontier Sciences of the Chinese Academy of Sciences (Grant QYZDY-SSW-JSC029), and K. C. Wong Education Foundation is gratefully acknowledged. The authors thank Dr. Ye Tian (TIPC) for her help in cytotoxicity experiment.

Keywords: Supramolecular chemistry • Self-assembly • Pure organic phosphorescence and fluorescence • Oxygen probe • Ratiometric detection

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COMMUNICATION

An organic hypoxia nanoprobe assembled by quadruplehydrogen bonds, shows efficient long-lived phosphorescence (Φ_P=7.7%, TP=3.2 ms) and fluorescence from a monochromophore at room temperature for oxygen detection in water and living cells. This is the first example to realize ratiometric hypoxia sensing by supramolecular assemblies of an organic monochromophore.



Ratiometric hypoxia detection from pure organic assemblies in water and living cells Xiao-Qin Liu⁺, Ke Zhang⁺, Jian-Feng Gao, Yu-Zhe Chen*, Chen-Ho Tung, and Li-Zhu Wu*

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Monochromophore-based

phosphorescence and fluorescence from pure organic assemblies for ratiometric hypoxia detection