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Imaging of cellular oxygen via two-photon excitation of fluorescent sensor nanoparticles

Xu-dong Wang[a], Daniela E. Achatz[a], Christina Hupf[a], Michaela Sperber[a], Joachim Wegener[a], Sebastian Bange[b], John M. Lupton[b], Otto S. Wolfbeis[a,*]

[a] Institute of Analytical Chemistry, Chemo- and Biosensors, University of Regensburg, 93040 Regensburg, Germany
[b] Institute of Experimental and Applied Physics, University of Regensburg, 93040 Regensburg, Germany

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ABSTRACT

Polystyrene nanoparticles (PSNPs) with an average size of 85 nm and loaded with an oxygen-quenchable luminescent ruthenium complex were used to sense and image oxygen inside cells following 2-photon excitation (2-PE). The ruthenium probe possesses a large two-photon absorption cross-section, and 2-PE is achieved by irradiation in the near infrared with commercially available fs-pulsed laser systems. The luminescence of the dye-loaded PSNPs is strongly quenched by oxygen, and Stern–Volmer plots are linear for both conventional single-photon excitation (1-PE) and for 2-PE. The particles do not show any significant cytotoxicity below a threshold concentration of 5 μg/mL and are readily taken up by mammalian cells (MCF-7), presumably via membrane mediated pathways. Thus, the PSNPs promise to be well suited to image the oxygen distribution in living cells and tissues. The 2-PE is considered to be advantageous over conventional imaging techniques because it works in the near-infrared where background absorption and luminescence of biomatter is much weaker than at excitation wavelengths below 600 nm.

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1. Introduction

Molecular oxygen is one of the most important species in chemical and life sciences [1]. For example, around 200 g of oxygen are metabolized by adults per day [2]. A lack of oxygen is closely related to various diseases [3], tissue and lung dysfunction, cancer growth [4], and of course can cause death if supply is interrupted for only 5–6 min. The determination of oxygen content in tissue provides essential information on understanding oxygen transport [5], tissue function and metabolism, malfunction and carcinogenesis, which – in turn – is beneficial for novel medical treatment [6–9]. Optical sensing of oxygen is superior to the electrochemical route due to its ease of use, the fact that oxygen is not consumed, its non-invasive nature, and – most importantly – the possibility of imaging oxygen over a relatively large area.

Optical sensing of oxygen relies on the excitation of oxygen-sensitive fluorescent probes using adequate light sources, but intracellular sensing is sometimes compromised by short-wavelength excitation because of poor tissue penetration and strong background luminescence of most biomatter [10]. The effect is worst in the UV but also significant in the blue part of the spectrum. Near infrared (NIR) light (650–900 nm) has deeper tissue penetration [11], but oxygen sensitive probes with excitation and emission wavelength in the NIR region are rare [12–16].

Such limitations have been overcome in the past by using two-photon excitation (2-PE) [17], but surprisingly there is no report so far on the use of ruthenium-based probes for 2-PE-based sensing of oxygen. However, 2-PE was used in combination with (dendritic) porphyrin-based phosphorescent probes for oxygen by the group of Vinogradov [18,19]. Later, Kondrashina et al. [20] have used a highly photostable platinum(II)porphyrin along with poly(9,9-dioctylfluorene) which acts as a Förster resonance energy transfer (FRET) donor and two-photon antenna, respectively, in another kind of 2-PE system. It also has been reported that NIR light can be used for sensing oxygen by making use of upconversion nanoparticles of the type $NaYF_4$ and doped with Yb(III) and Tm(III) that can act as light sources ("nanolamps") that convert NIR light (980 nm) into visible light via sequential 2-photon absorption. The emission of the upconversion nanoparticles contained in a sensor film was used to photoexcite an iridium complex whose luminescence is quenched by oxygen [21]. This represents an indirect way to photoexcite the oxygen sensitive probes. Direct 2-PE of chromophores, in contrast to the sequential photoexcitation of upconversion nanoparticles, relies on the almost simultaneous absorption of two photons and

* Corresponding author. Tel.: +49 941 943 4066; fax: +49 941 943 4064.
E-mail addresses: otto.wolfbeis@ur.de, Otto.Wolfbeis@chemie.uni-r.de (O.S. Wolfbeis).
URL: http://www.wolfbeis.de (O.S. Wolfbeis).

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can be accomplished efficiently only using pulsed lasers in which the photons are packed in short temporal intervals.

Direct 2-PE of ruthenium probes has not been applied to optical sensing and imaging of oxygen so far despite previous reports that ruthenium diimine complexes undergo efficient 2-PE [22–26]. We perceived that 2-PE in the near infrared may be particularly beneficial in terms of intracellular sensing of oxygen, not the least in view of a report that a poly(ethylene glycol)-decorated ruthenium(II) complex can be used for two-photon photodynamic therapy [27]. The use of an oligo- or poly(ethylene glycol) coating makes such nanosensors viable probes for intracellular sensing because such coatings warrant biocompatibility and non-cytotoxicity. Other applications of 2-PE include the use of a ruthenium polypyridine cage complex to cause the photorelease of glutamate if photoexcited with 800 nm light via 2-PE [28], and a ruthenium(II) dye was shown to act as a photoinitiator in the two-photon induced fabrication of gold microstructures in thin films of polystyrene sulfonate [29]. It is noted here that 2-PE was used in fluorescence microscopy to investigate the size and crystallinity of microcrystals in polymer-supported oxygen sensors, but not to sense oxygen [30]. It is generally known that 2-PE enables particularly high contrast imaging to be performed [31].

Most of the currently used oxygen sensitive probes are of the metal-ligand complex type [12,13]. These probes have very low two-photon absorption (TPA) cross-sections in the order of a few Goeppert-Meyer (GM) units (where 1 GM equals 10^{-50} cm^4 s/photon). In order to significantly increase their TPA cross-sections, light-harvesting antennas were co-immobilized with oxygen sensitive probes to form nanoparticle-based sensors [28–34]. They usually employ small organic fluorescent molecules or fluorescent nanocrystals with high molar absorbance that efficiently capture two-photon energy. However, efficient energy transfer requires the oxygen sensitive probes and the antennas to have good spectral overlap and to be spatially close. Still, the TPA cross-sections have remained below 100 GM [32].

It was reported that the $Ru(dpp)_3$ complex (where dpp stands for 4,7-diphenylphenanthroline) has a particularly high TPA cross-section of 210 GM [33]. It is anticipated that oxygen probes derived from certain ruthenium diimine complexes may be favorable for optical sensing schemes based on 2-PE. This paves the way to imaging of oxygen in cells and tissues by directly exciting these probes via 2-PE, and results in high-resolution images as will be shown below.

2. Experimental

2.1. Materials

Amino-modified polystyrene nanoparticles (PSNPs; from Polybead®; type Amino 0.10 μm spheres; a 2.6%-suspension of solid particles in water) were purchased from Polysciences, Inc. (www.polysciences.com). The oxygen probe ruthenium(II) tris(4,7-diphenyl-1,10-phenanthroline) dichloride was made polystyrene-soluble by converting it into its di(trimethylsilylpropanesulfonate) salt $[Ru(dpp)_3]TMSPS_2$ according to a known method [34,35]. It is also available commercially.

2.2. Instruments

Confocal laser scanning microscopy (CLSM) images of cell layers loaded with sensor particles (referred to as Ru-PSNPs) were recorded using a Nikon Eclipse 90i with the confocal unit C1. A Spectra Physics 2045E cw argon laser (www.newport.com) in combination with a Semrock LL01-488 (www.semrock.com) laser clean up filter was used for one-photon excitation (1-PE) at a wavelength of 488 nm and a power of 430 μW. The 2-PE experiments were performed with a Spectra-Physics Tsunami mode-locked Ti:sapphire laser with a nominal repetition rate of 80 MHz, a wavelength of 830 nm and an average power of 130 mW. The average pulse duration of the latter was 75 fs as determined using an APE PulseCheck autocorrelator (www.ape-berlin.de). The direction of polarization was vertical in the mutually orthogonal excitation and detection pathways.

In a typical experiment, the laser beam was directed through a quartz cuvette to which the particle solutions (400 μL each) were added with a syringe. The red luminescence (peaking at around 635 nm) was detected by a 300 mm focal length grating spectrometer and CCD camera (www.princetoninstruments.com) with an integration time of 20 s. A-488 nm long-pass filter (www.semrock.com) was used to block the scattered laser light in case of 1-PE, and a Semrock FF01-680/SP short-pass filter in the case of 2-PE.

2.3. Synthesis of the oxygen-sensitive nanoparticles

The Ru-PSNPs sensor particles were obtained by staining the PSNPs with $[Ru(dpp)_3](TMSPS)_2$ via a soaking procedure according to a previously published protocol [35]. Typically, 500 μL of the aqueous particle suspension were diluted with 2.0 mL of distilled water. The probe $[Ru(dpp)_3](TMSPS)_2$ (1.0 mg) was dissolved in 100 μL of tetrahydrofuran and the solution was added to the suspension of the PSNPs. The mixture was treated in an ultrasonic water bath for 1 min before removing the tetrahydrofuran using a rotary evaporator (at 220 mbar, room temperature) over a period of 2 h. The nanoparticle concentration in the remaining (mainly aqueous) suspension was determined to be $c = 4.0$ mg/mL by centrifugation and weighing. The ruthenium probe is almost completely incorporated into the particles because after centrifugation the supernatant is virtually non-fluorescent and colorless. The number of nanoparticles per volume n was calculated according to the following equation [36]:

$$n = \frac{6 \cdot c}{\rho \cdot \pi \cdot d^3} \quad (1)$$

With the density of polystyrene $\rho = 1.05$ g/cm^3, an average diameter of $d = 85$ nm and the mass of particles per volume c, the calculation yields a value of $n = 1.2 \times 10^{13}$ mL^{-1}. This orange-colored stock suspension of sensor particles was stored in the dark at 4 °C.

2.4. Sensing oxygen by 1-PE and 2-PE spectroscopy

The aqueous suspension containing 0.4 wt% sensor particles was depleted of oxygen by bubbling it with a flow of argon gas for 30 min. Synthetic gas mixtures were prepared using a custom gas mixing device. Response to oxygen was measured in cuvettes filled with suspensions of sensor particles. A flow of a synthetic argon/oxygen mixture (80/20) was passed through the cuvette via a flexible tube and a cannula, and 400 μL of the deoxygenated particle solution was then added using a syringe. After 5 min of further bubbling with argon/oxygen (80/20), luminescence spectra were recorded under both 1-PE and 2-PE. Then, argon gas containing oxygen in varying fractions ($Ar:O_2$ ratios of 80:20; 60:40; 40:60; 20:80; 0:100) were passed through the cuvette for 10 min, respectively, and luminescence spectra were recorded again.

2.5. Interaction of Ru-PSNPs with animal cells

All experiments addressing the interaction of nanoparticles with mammalian cells were performed using the human breast

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