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Phosphorescent nanoparticles for quantitative measurements of oxygen profiles *in vitro* and *in vivo*

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Abstract

We present the development and characterization of nanoparticles loaded with a custom phosphor; we exploit these nanoparticles to perform quantitative measurements of the concentration of oxygen within three-dimensional (3-D) tissue cultures *in vitro* and blood vessels *in vivo*. We synthesized a customized ruthenium (Ru)-phosphor and incorporated it into polymeric nanoparticles via self-assembly. We demonstrate that the encapsulated phosphor is non-toxic with and without illumination. We evaluated two distinct modes of employing the phosphorescent nanoparticles for the measurement of concentrations of oxygen: 1) *in vitro*, in a 3-D microfluidic tumor model via ratiometric measurements of intensity with an oxygen-insensitive fluorophore as a reference, and 2) *in vivo*, in mouse vasculature using measurements of phosphorescence lifetime. With both methods, we demonstrated micrometer-scale resolution and absolute calibration to the dissolved oxygen concentration. Based on the ease and customizability of the synthesis of the nanoparticles and the flexibility of their application, these oxygen-sensing polymeric nanoparticles will find a natural home in a range of biological applications, benefiting studies of physiological as well as pathological processes in which oxygen availability and concentration play a critical role.

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Keywords

Nanoparticle; Oxygen-sensing; Ruthenium phosphor; Poly(urethane acrylate nonionomer) (PUAN); Tissue engineering; Vascular oxygen concentration

1. Introduction

Spatiotemporal variation of the concentration of oxygen in tissue plays a central role in regulating biological processes, including embryonic [1,2], vascular [3] and bone [4] development, as well as stem cell function [5,6] and wound healing [7]. Diseases that involve these processes, such as cancer, are also strongly regulated by the distribution and concentration of oxygen in tissue [8,9]. Tools to measure profiles of oxygen with micrometer-scale resolution in three-dimensional (3-D) systems could help improve basic understanding of the role of oxygen in a range of both physiological and pathological processes; to date, few such tools are available. Standard approaches include immunohistochemical staining [10], electrochemical measurements [11,12], and optical techniques [13-16]. These techniques are challenging to integrate within biological systems, are often semi-quantitative, invasive, or do not provide a sufficient spatial and temporal resolution to capture physiologically relevant variations of oxygen concentration.

Imaging of oxygen via measurement of phosphorescence quenching provides a non-invasive means of monitoring the concentration of dissolved molecular oxygen in biological systems, both *in vitro* and *in vivo* [13]. In 2-D, this technique is widely accessible in the form of commercially available oxygen-sensitive patches [17] that can be incorporated into traditional 2-D cell culture formats [15]. More recently, Abaci, *et al.* have demonstrated the measurement of oxygen concentration on the bottom of 3-D cellular scaffolds using SensorDish Reader [16] as an important cell culture tool. Hurdles to the use of quantitative phosphorescence imaging as a generic tool for a wide range of tissue-scale applications include complexity and cost of synthesis of materials, biological toxicity presented by the hydrophobic phosphors themselves, and by singlet oxygen or superoxide production, which are natural byproducts of optical probes of oxygen. Encapsulation of dyes in dendrimers [18-21] provides an approach to avoid direct cellular contact with toxic phosphors, but these structures are chemically complex and can be both expensive and challenging to synthesize. Widely used phosphors are based on platinum (Pt), which has an intrinsically low cross-section for two-photon absorption and a relatively long lifetime; the combination of these two characteristics has led to signals that are inherently dim, often too noisy to get quantitative data. To combat this problem, Finikova, *et al.* demonstrated a Pt-dendrimer complex with antenna molecules that enhanced the photon absorption cross-section significantly [19]. Loading of dyes within microparticles [22] offers a simpler alternative for encapsulation, but the large size of the particles presents problems with regards to optical scattering and perturbation of the mechanical and optical characteristics of the medium in which they are dispersed (e.g., a tissue scaffold or blood within capillary-sized vessels).

Here, we report on the synthesis and applications of new oxygen-sensing nanoparticles for quantitative imaging of the distribution of oxygen *in vitro* and *in vivo*. We synthesized polymeric nanoparticles containing an oxygen-sensitive phosphor and demonstrate that this encapsulation approach eliminates the toxicity presented by freely dissolved phosphor. We employed a ruthenium (Ru)-based phosphor with a moiety that interacts with the excited state of the transition metal; this structure can be used to tune the phosphorescence lifetime to an optimal balance between good oxygen sensitivity and reasonable imaging speed. Ru-complexes exhibit a significantly higher cross section for two-photon absorption compared with other phosphors used for biological imaging, and therefore required no additional

enhancement or amplification [18,19,21] (also see Supplementary Content). We evaluate multiple options for quantification of oxygen concentration: i) direct phosphorescence intensity, ii) phosphorescence intensity normalized by that of an oxygen-insensitive reference fluorophore, and iii) phosphorescence lifetime. We worked in both 3-D scaffolds seeded with tumor cells and cultured *in vitro*, and in the blood within an artery and vein of live mice *in vivo*. We discuss outstanding challenges and opportunities for the application of these particles and optical methods to a range of biological systems.

2. Materials and methods

2.1. Quantification of oxygen concentration from optical measurements

The concentration of oxygen was extracted from optical measurements (i.e., intensity or lifetime) using the Sterne–Volmer relationship [23,24]. Assuming that quenching is the only process that affects the luminescence decay of the phosphor, the ratio between the luminescence lifetime (or integrated luminescence intensity) in the absence and presence of quenching molecules (oxygen molecules in this study) is linearly proportional to the concentration of the quencher:

$$\frac{\tau_0}{\tau} = \frac{I_{\text{phos},o}}{I_{\text{phos}}} = 1 + K_{\text{SV}} c_{\text{O}_2} \quad (1)$$

where τ [s] is phosphorescence lifetime. The subscript 0 denotes the absence of oxygen. I_{phos} is phosphorescence intensity, K_{SV} [M^{-1}] is Sterne–Volmer constant or oxygen sensitivity, and c_{O_2} [M] is concentration of dissolved oxygen. Note that Eq. (1) describes the ideal, theoretical situation where the quenching undergoes by bimolecular collisions under identical conditions (i.e., the same illumination and molecular environment of the phosphor). Sterne–Volmer constants experimentally estimated from our intensity-based measurements (K_{SV}^I) and lifetime-based measurements (K_{SV}^τ) were not the same but of the same order of magnitude (for more details, also see Results and Discussion: Section 3.5 and Supplementary Fig. S3).

2.2. Synthesis of oxygen-sensitive phosphor, [(TEG-bpy)₂Ru(bpy-pyr)]Cl₂ (Ru-TEG)

First, a reaction of two equivalents of the triethyleneglycol-bipyridine (TEG-bpy; bis-(4-methyl-4'-(triethyleneglycolmethylether)methylethyl-2,2'-bipyridine) ligand with RuCl₃ in 2-methoxyethanol (CH₃OCH₂CH₂OH) produced the precursor, [(TEG-bpy)₂RuCl₂]. The reaction was run under argon with citrate as a reducing agent. The ligand was prepared by the published method developed by Kuang *et al.* [25]. TEG-bpy (0.64 g, 1.8 mmol), RuCl₃·3H₂O (0.24 g, 0.9 mmol), and LiCl (0.4 g, 9.4 mmol) were dissolved in 25 mL of degassed 2-methoxyethanol, and the mixture was refluxed under argon for 16 h. At this point, ascorbic acid (160 mg, 0.85 mmol) was added to the brown mixture, and was refluxed for another 6 h whereupon the solution had turned dark purple. The solvent was evaporated until near dryness, diluted with water (50 mL) and filtered. The filtrate was extracted into methylene chloride (CH₂Cl₂; 3 × 30 mL), dried over anhydrous sodium sulfate (Na₂SO₄), and evaporated to dryness to yield 0.385 g (49%) of purple oil. The product was used as is in preparation of the bipyridylpyrene complex.

The precursor was then refluxed under argon with bpy-pyr (4-(1-pyrenyl)-2,2'-bipyridine) in chloroform (CHCl₃)/ethanol (C₂H₅OH) (1:2 by volume) to yield the product complex, [(TEG-bpy)₂Ru(bpy-pyr)]Cl₂ (Fig. 1a) as the chloride salt. [(TEG-bpy)₂RuCl₂] (380 mg, 0.44 mmol) and pyr-bpy (156 mg, 0.44 mmol) were dissolved in degassed chloroform/ethanol (1:2, 30 mL), one drop triethylamine (N(CH₂CH₃)₃) was added, and the mixture was

then stirred and refluxed under argon for 16 h until the solution became dark red. Thin layer chromatography (TLC) on silica showed three spots. The product red solid (185 mg, yield: 34.4%) was obtained after purification by column chromatography on alumina eluting with methylene chloride: ethyl acetate ($\text{CH}_3\text{COOC}_2\text{H}_5$) : methanol (CH_3OH) (6:4:1).

Electrospray ionization-time of flight (ESI-TOF) mass spectral data are presented in Supplementary Content. The complex contained a small amount of an impurity in which one of the TEG-bpy ligands was substituted by 4,4'-dimethyl-2,2'-bipyridine (dmb), $[(\text{TEG-bpy})(\text{dmb})\text{Ru}(\text{pyr-bpy})]^{2+}$. We note that the photophysical behaviors of $[(\text{TEG-bpy})_2\text{Ru}(\text{bpy-pyr})]\text{Cl}_2$ and $[(\text{TEG-bpy})(\text{dmb})\text{Ru}(\text{bpy-pyr})]\text{Cl}_2$ are very similar (also see Supplementary Table S1).

2.3. Synthesis of amphiphilic urethane acrylate nonionomer (UAN)

The synthesis of UAN chain was carried out through a 3-step process as presented previously [26,27]. Each reaction was performed in a 500 mL 4-neck vessel with stirrer, thermometer, and an inlet for nitrogen gas. Step 1: hydroxyl groups of polypropylene oxide triol (PPO triol; MW 700) reacted with NCO groups of toluene diisocyanate (TDI) to form NCO-capped intermediate precursor chain (chain A). Step 2: two of NCO groups of chain A reacted with hydroxyl groups of 2-hydroxyethyl methacrylate (2-HEMA) to introduce reactive vinyl groups to precursor chains (chain B). Step 3: polyethylene glycol (PEG; MW 1500) was added, and it reacted with residual NCO groups of chain B. Molar ratio of TDI/PPO triol/2-HEMA/PEG was 3/1/2/1.

2.4. Synthesis of oxygen-sensing nanoparticles

Oxygen-sensing nanoparticles were synthesized via chemical crosslinking polymerization (Fig. 1). In detail, the phosphor was first partitioned in UAN consisting of polyethylene oxide (PEO; MW 1,500 g/mol) and polypropylene oxide (PPO; MW 700 g/mol): 1) 4 μmol of Ru-TEG was dissolved in 3 mL of methylene chloride, 2) 1 g of UAN was dissolved in the phosphor-dissolved methylene chloride, and 3) methylene chloride was allowed to evaporate in a fume hood for ~ 8 h. The UAN partitioned with Ru-TEG partitioned UAN was mixed with 9 mL of a solution of potassium persulfate (KPS; radical initiator) dissolved (2 mg/mL) in deionized (DI) water, thereby dispersing nanoparticles in water (Fig. 1c). Then, the cores of the UAN nanoparticles loaded with Ru-TEG were crosslinked (Fig. 1d) via conventional emulsion polymerization process with stirring (650 rpm) in a 65 $^\circ\text{C}$ silicone oil bath for 5 h; nitrogen gas was directly bubbled into the reaction medium for the first 2 h; for the remaining 3 h the reaction medium was agitated without bubbling nitrogen. The suspension of crosslinked nanoparticles (i.e., 10% [w/v] Ru-TEG-PUAN) in water was first filtered through syringe-filters (pore size: 200 nm), and then dialyzed with a molecular weight cutoff of 50 kDa for 2 days in order to eliminate uncrosslinked UAN and Ru-TEG molecules that were not incorporated into the PUAN. The 10% [w/v] refers to the weight/volume ratio of uncrosslinked Ru-TEG-UAN (solid) in water as measured prior to crosslinking. To incorporate coumarin540 (c540; Exciton) as a reference fluorophore after crosslinking of the Ru-TEG-PUAN particles, we proceeded as follows: 1) 120 μL of 2.28 mM c540 (274 nmol; MW 350.44 g/mol), predissolved in methylene chloride, was added into 10 mL of 10 fold-diluted Ru-TEG-PUAN stock suspension (i.e., 1% [w/v]) in water, and 2) the mixture was strongly agitated at 1,500 rpm for 24 h, followed by the same dialysis step as described above. Ru-TEG-c540-PUAN nanoparticles dispersed in water were stored at room temperature prior to use.

2.5. Measurement of size distribution of oxygen-sensing nanoparticles

The size distribution of oxygen-sensing nanoparticles dispersed in water was measured by Dynamic Light Scattering (DLS), using a Zetasizer Nano-ZS (Malvern Instruments). 400 μL

of each dispersion at a concentration of 1% [w/v] was used for measurements and analyzed with a 4 mW He–Ne laser (633 nm) to avoid unnecessary excitation of the phosphor that might affect DLS measurements. Refractive indices of 1.454 and 1.330 were used for PUAN and water, respectively. Temperature effects on the size distribution were examined by using viscosities of 0.8872 and 0.6864 cP for water at 25 and 37 °C, respectively (see Table 1). A total of 6 measurements were performed on each dispersion, and each measurement consisted of 12 repeated sub-runs. The average diameter, Z_D and polydispersity index (PDI) representing size distributions were computed automatically in Zetasizer software (www.malvern.com). The PDI is the relative variance of the DLS-based size distribution, defined as σ^2/Z_D^2 , where σ is standard deviation of the size distribution; for example, polydispersity indices ranging from 0.08 to 0.7 represent a mid-range polydispersity (www.malvern.com).

2.6. UV–Vis emission spectra and long-term stability of oxygen-sensing nanoparticles

UV–Vis emission spectra of Ru-TEG and Ru-TEG-c540-PUAN in water were measured using a Cary 300 UV–Vis spectrophotometer. Each sample was loaded in a quartz cuvette, and deoxygenated by bubbling nitrogen gas for 10 min immediately before the measurements.

We tested whether incorporated luminophores (specifically, Ru-TEG) leaked out of PUAN to water over a long period of time (i.e., several months), by measuring emission spectra of 1) DI water, 2) solvent eluted from a spin column filter (3 kDa cutoff) upon centrifuging a 0.5% [w/v] dispersion of Ru-TEG-c540-PUAN nanoparticles in water at ambient oxygen level (i.e., 21% O₂), 3) solvent eluted from the same dispersion, but measured under deoxygenated conditions, 4) 0.25% [w/v] Ru-TEG-c540-PUAN dispersed in water at ambient oxygen level, and 5) deoxygenated Ru-TEG-c540-PUAN dispersed in water (Fig. 2b and Fig. 3). The positive control, 0.25% [w/v] Ru-TEG-c540-PUAN nanoparticles, was re-dialyzed immediately before the measurements; the nanoparticles had been dispersed in water for ~10 months after synthesis and initial dialysis.

2.7. 3-D culture of tumor cells seeded in alginate discs containing dispersed oxygen-sensing nanoparticles

Sterile-filtered 1% [w/v] Ru-TEG-c540-PUAN nanoparticles dispersed in water were mixed with phenol red-free Dulbecco's Modified Eagle Medium (DMEM; Invitrogen): 1:1 volume ratio, resulting in 0.5% [w/v] Ru-TEG-c540-PUAN nanoparticles. This solution was then used to dissolve lyophilized alginate (Protanal LF 10/60; FMC Biopolymer) to a final alginate concentration of 4% [w/v]. Then, oral squamous cell carcinoma cells (OSCC-3; received as a gift from Peter Polverini, University of Michigan) were gently suspended in the alginate (2×10^7 cell/mL). Calcium alginate discs (200 μ m thick, 4 mm in diameter) were fabricated as described previously [9]: molded alginate was crosslinked with 60 mM CaCl₂. The thickness of the alginate discs was chosen to avoid oxygen heterogeneity throughout the disc cross-section, as previously confirmed [9]. Alginate discs seeded with both OSCC-3s and oxygen-sensing nanoparticles were cultured in DMEM with 10% [v/v] fetal bovine serum (FBS; Tissue Culture Biologicals) and 1% [v/v] penicillin/streptomycin (PS; 120 U/mL; Gibco) at 37 °C, 5% CO₂ for 7 days. Media was changed daily through the culture period.

2.8. Toxicity testing

OSCC-3-seeded alginate discs both with and without the oxygen-sensing nanoparticles were exposed to blue-violet light via a custom filter set (Chroma Technology; excitation 425 nm; emission 630 nm; 460 nm dichroic splitter) for 10 and 30 s daily for 7 days during the 3-D

culture. The same exposure scheme was applied while OSCC-3-seeded alginate discs without Ru-TEG-c540 nanoparticles were cultured in media containing free Ru-TEG, at a concentration comparable to that in discs with dispersed nanoparticles. Assuming 100% incorporation of 4.5 μmol of Ru-TEG (MW 1150.3301 g/mol) in 10 mL of 10% [w/v] PUAN nanoparticles after synthesis, the concentration of free Ru-TEG in culture media was calculated based on the nanoparticle concentration in alginate (0.25% [w/v]): $(4.5 \text{ mmol}/10 \text{ mL}) \times (0.25\%/10\%) = 112.5 \text{ nM}$. As controls, the alginate discs were also cultured without the excitation light exposure.

2.9. Live/dead staining

Viability of OSCC-3s seeded in alginate discs, with and without oxygen-sensing nanoparticles, free phosphor and light exposure, was estimated using live-dead staining. After 7 days in culture, alginate discs were submerged in a live/dead staining solution (5 μM calcein-AM and 5 μM ethidium homodimer-1; Invitrogen) in a pH-adjusted buffer solution. The submerged discs were incubated for 1 h prior to fluorescence microscopy. Images were taken on an inverted microscope (Axio Observer; Carl Zeiss). ImageJ was used to merge green and red fluorescence images.

2.10. DNA assay

Alginate discs were first dissolved in 50 mM ethylenediaminetetraacetic acid (EDTA, EMD Chemicals). Released cells were rinsed with phosphate buffered saline (PBS) after centrifugation, and then lysed in Caron's buffer. DNA content was measured using Quant-iTTM PicoGreen[®] dsDNA reagent (Invitrogen) according to manufacturer's instructions, and normalized to day 1 control values.

2.11. Fabrication of cell-seeded scaffolds for optical measurement of oxygen-depletion lengths

To allow for the generation of one-dimensional (1-D) gradients of oxygen, a microfluidic channel was fabricated adjacent to calcium alginate seeded with cells. The process was adapted from that described previously [28]: 1) a machined plexiglass mold (4 mm in length $\times$ 4 mm in width $\times$ 0.5 mm in depth) was pre-coated with 1% [v/v] polyethylenimine (PEI) to allow for the adhesion of alginate, 2) alginate dispersed with both OSCC-3s (5×10^6 or 2×10^7 cell/mL) and Ru-TEG-c540-PUAN nanoparticles (0.5% [w/v]) was cast in the machined plexiglass mold, with an aluminum plug inserted to block the channel, and 3) a machined plexiglass cover was placed to pressure-seal the channel. The aluminum plug was replaced with a gas-impermeable polyisobutylene rubber plug (McMaster-Carr) when sealing the device (Fig. 4a). Note that the cells were dispersed throughout the bulk of the 3-D scaffold. In this section, '1-D' refers to the uniaxial variation in the distribution of oxygen, perpendicular to the channel (y -direction in Fig. 4b); this condition was imposed by maintaining constant concentration of oxygen via flow along the channel direction (z -direction in Fig. 4b) and through the thickness of the scaffold (x -direction in Fig. 4b). The microfluidic system was used for 1) the optical measurement of oxygen-depletion lengths and 2) calibration of Ru-TEG-c540-PUAN nanoparticles dispersed in alginate.

2.12. Visualization and optical measurement of oxygen-depletion lengths in a microfluidic tumor model, in vitro

Fluorescence and phosphorescence images of cell-seeded scaffolds were taken at $4 \times$ magnification (field of view: $2.1 \times 1.6 \text{ mm}$) with 2×2 pixel binning and a customized filter cube (excitation at 392 nm and phosphorescence emission at 650 nm; Chroma Technology) for Ru-TEG, and a GFP filter cube for c540 on an inverted microscope (IX 81, Olympus), a CCD camera (Hamamatsu), and Slidebook software for image acquisition (Intelligent

Imaging Innovations). The stage of the microscope was contained within an environmental chamber (Weather Station, Precision Control) to maintain a humid environment at 37 °C for the duration of the imaging. In order to capture the distribution of oxygen with its diffusive transient, phosphorescence and fluorescence images were acquired every minute for 45 min while phenol red-free DMEM was delivered via the single channel adjacent to the alginate slab in which both OSCC-3s (5×10^6 or 2×10^7 cell/mL) and Ru-TEG-c540-PUAN nanoparticles were dispersed. Background images, representing dark-current, were acquired separately for a given exposure time (0.5 s) by blocking the light path to the CCD camera. Acquired images were then background-subtracted, analyzed and color-mapped using Matlab (Mathworks); phosphorescence and fluorescence intensities were averaged along the microchannel direction (Fig. 4b), and the ratio ($\bar{I}$) of phosphorescence of Ru-TEG (I_{phos}) to reference fluorescence of c540 (I_{fluor}) was calculated as an un-calibrated measure of oxygen concentration.

2.13. Intensity-based calibration of oxygen-sensing nanoparticles

To calibrate the ratio of intensities (i.e., $\bar{I} = I_{\text{phos}}/I_{\text{fluor}}$), the plexiglass cover (as shown in Fig. 4a) was removed from cultures. Scaffolds were then fixed with 3.7% [w/v] formaldehyde in order to eliminate any gradients of oxygen concentration through the scaffold thickness resulting from cellular consumption in a top-fed configuration. The ratio of intensities was calibrated *in situ* with delivery of 10 mM CaCl₂ buffer solution at dissolved oxygen concentrations of 0, 65, 130, 195, and 273 μM (i.e., buffer solution saturated with partial pressures of oxygen of 0, 5, 10, 15, and 21%), over the top of the scaffold, using a peristaltic pump (Watson-Marlow). The CaCl₂ in the buffer was required to ensure the rigidity of the crosslinked alginate scaffolds [28]. A high flow rate (i.e., ~ 0.5 mL/min) was used to maintain the desired oxygen level at the device (verified independently with oxygen electrode measurements; data not included). The desired concentration of dissolved oxygen was achieved by directly bubbling a blend of air and pure nitrogen, controlled by a HypoxyDial (STARR Life Sciences). The scaffolds were allowed to equilibrate at each oxygen concentration for 20 min (Fig. 5a and b, Fig. 6).

Non-ratiometric calibration data (only I_{phos} values from the calibration experiments described above) were also used to generate a Stern–Volmer plot (also see Fig. 5c).

2.14. Finite element modeling (FEM) of diffusion and reaction of oxygen within cell-seeded scaffolds

We carried out FEM assuming that the spatially resolved distributions of oxygen concentration at steady-state are governed by Michaelis–Menten kinetics and Fickian Diffusion:

$$D_{\text{O}_2} \nabla^2 c_{\text{O}_2} = \frac{(\rho_{\text{cell}} V_{m,\text{cell}}) c_{\text{O}_2}}{K_M + c_{\text{O}_2}} \quad (2)$$

where $V_{m,\text{cell}}$ represents maximum rate of cellular oxygen consumption, and K_M [M] is Michaelis–Menten constant. We used 5.4×10^{-17} mol/s-cell for $V_{m,\text{cell}}$; this value was measured independently for OSCC-3 cells in alginate [9]. We used K_M as an adjustable parameter to generate computationally calculated profiles of oxygen concentration shown in Fig. 7b (dark red and dark blue solid lines). By iterating the computational calculation to aim for a square correlation coefficient (R^2) greater than 0.95, a single value of $K_M = 12.3 \mu\text{M}$ was selected for the two cell seeding densities.

2.15. Two-photon optical calibration of oxygen-sensing nanoparticles by measurement of phosphorescence lifetimes and two-photon absorption cross-sections

Oxygen-sensing nanoparticles dispersed in water were also calibrated by measuring phosphorescence lifetime. As described above, dissolved oxygen concentration was controlled using an oxygen/nitrogen mixer, with a dynamic range from 0 to 100% O₂, OxyDial (STARR Life Sciences). Lifetime measurements were made on a two-photon microscope consisting of a Ti:Sapphire laser (Millennia Xs/Tsunami combination, Spectra Physics) directed into a modified BioRad MRC 600 laser scanner interfaced with a modified (fixed-stage) upright microscope (AX-70, Olympus). A 350-80 BKLA Pockel's Cell (Conoptics) with custom-made electronics provided modulation of beam intensity and blanking during scanner flyback. 780 nm pulsed excitation light was focused into the specimen with a large-barrel Olympus 20x/0.95 NA water-immersion dipping objective lens. Emission was separated with a 750 nm long-pass dichroic, filtered with a HQ650/100M emission filter (both from Chroma Technology) and detected with a GaAsP PMT (H7422P-40, Hamamatsu), amplified, and directed into the external ports of the BioRad acquisition electronics.

The Pockel's Cell was used to deliver repeated 5 μ s-pulses followed by monitoring periods with no laser power (<3% corresponding to < 0.1% excitation). Phosphorescence curves were acquired using a SR430 multichannel scaler (Stanford Research Systems) and the electronics were synchronized for repeated patterns using a DG535 digital delay/pulse generator (Stanford Research Systems, 20,000 reps at 1 kHz for each curve). The decay curves were fit to single exponentials using Origin (Origin Lab). More detailed information about the multi-photon microscopy can be found from work done by Williams *et al.* [29].

Optical cross-section measurements were carried out by comparison to rhodamine B as previously described [30]. Briefly, phosphorescence/fluorescence photons were counted from Ru-TEG and standard solutions, ensuring an intensity squared dependence with no effects from molecular saturation or photobleaching. The slopes of these lines (i.e., signal to squared intensity) are an indication of molecular brightness. They were corrected for the emission lineshape, the spectra lineshape of the filters and detectors, and the concentration and quantum yields of the measured solutions.

2.16. In vivo oxygen imaging in mouse saphenous vasculature

Female FVB mice (4–6 week old) were anesthetized with 1–2 % isoflurane gas adjusted to maintain a breathing rate of 100 breaths/min. Unless otherwise stated, the carrier gas for the anesthesia was pure oxygen. Each mouse was positioned in dorsal recumbency on one side of the stage on top of a heating pad with a rectal feedback probe (FHC) set to maintain core temperature at 36 °C. All animal procedures were performed according to Cornell animal health protocols [2007-0179 and 2004-0030]. The left limb was extended into a separate chamber for perfusion with saline, and a minor incision through the skin was made to expose the saphenous vessels, leaving the fascia intact. Once positioned under the microscope, the extended hindlimb was perfused with warm saline, and the breathing rate and core temperature were continuously monitored to assure physiological stability (also see Fig. 8b). Several minutes before imaging the mouse received an intracardiac injection with Ru-TEG-PUAN nanoparticles (3 μ L/g mouse weight).

The vasculature was imaged with a custom-built multi-photon microscope described previously [29] and above. Excitation light was focused into the vasculature using an Olympus 20X/0.95NA water-immersion objective that provided a large field of view and several millimeters of necessary working distance. The illumination wavelength was tuned to 780 nm and nonlinear emissions were collected in epifluorescence mode and separated

from the excitation beam using a 750DCXRU long-pass dichroic filter (Chroma Technology). The resulting nonlinear emissions were spectrally split by a 560DCXRU into green-(BGG22 + 525/50 emission filters, Chroma Technology) and red- (650DF100MP, Chroma Technology) pseudocolored channels. The green emission (c540 fluorescence and some autofluorescence) was detected with a bi-alkali PMT (HC125-02, Hamamatsu) and the Ru-TEG emission was detected using a red-sensitive GaAsP photocathode PMT (H7422P-40, Hamamatsu) with custom-designed amplifier circuitry. Lifetime measurements were carried out by zooming the beam to a $12 \times 12 \mu\text{m}$ field-of-view at positions of interest and proceeding as described in the previous section. Multiphoton images were acquired by optically sectioning at $20 \mu\text{m}$ intervals with a 4x objective lens. Image analysis was accomplished with custom routines written in the IDL image analysis environment (ITT Visual Information Solutions, Boulder Co).

2.17. Statistics and estimation of propagated uncertainties

Plots shown are mean $\pm$ standard deviation. Statistical significance was assessed using Student's *t*-test and 1-way analysis of variance (ANOVA). $p < 0.05$ was considered significant.

For uncertainties in the master calibration, we considered 6 independent measurements of the ratiometric intensity ($\bar{I}$) at each oxygen concentration (c_{O_2}): 3 for 5×10^6 cell/mL and 3 for 2×10^7 cell/mL. I_{phos} and I_{fluor} are background-subtracted mean intensities of Ru-TEG and coumarin540 respectively over $672 \times 512 = 344064$ pixels per each measurement. The background signal is averaged current noise from the CCD camera. From 6 measured intensities at $0 \mu\text{M O}_2$, $\bar{I}_0 = 5.45 \pm 1.04$ (mean $\pm$ standard deviation). Since $\bar{I}$ and $\bar{I}_0$ are correlated, uncertainty of $\bar{I}$ is [31]

$$\delta \bar{I} = \sqrt{(\delta I)^2 \left(\frac{\bar{I}}{I} \right)^2 + (\delta I_0)^2 \left(\frac{\bar{I}}{I_0} \right)^2 + 2\delta_{I_0} \left(\frac{\bar{I}}{I} \right) \left(\frac{\bar{I}}{I_0} \right)}$$

$$\text{where } \bar{I}_{I_0} = \frac{1}{N} \sum_{i=0}^N (I_i - I) (I_{0,i} - I_0), \frac{\bar{I}}{I} = -\frac{I_0}{I^2} \text{ and } \frac{\bar{I}}{I_0} = \frac{1}{I}$$

After the linear regression between $\frac{\bar{I}_0}{I}$ and c_{O_2} with 1 parameter fit (*y*-intercept fixed to 1; slope $K_{\text{sv}} \bar{I}$ varied; individual data points weighted by the uncertainty), the half 95% confidence interval of $K_{\text{sv}} \bar{I}$ was used as uncertainty in $K_{\text{sv}} \bar{I}$.

For uncertainties in c_{O_2} , we propagated uncertainties through the linear Stern–Volmer calibration:

$$c_{\text{O}_2} = \frac{1}{K_{\text{sv}} \bar{I}} \left(\frac{\bar{I}_0}{I} - 1 \right)$$

The uncertainties in c_{O_2} at each position along *y*-axis were calculated using

$$\frac{c_{O_2}}{K_{sv} \bar{I}} = -\frac{1}{\left(K_{sv} \bar{I}\right)^2} \left(\frac{\bar{I}_0}{\bar{I}} - 1\right), \frac{c_{O_2}}{\bar{I}_0} = \frac{1}{\bar{I} \cdot K_{sv} \bar{I}}, \frac{c_{O_2}}{\bar{I}} = -\left(\frac{\bar{I}_0}{K_{sv} \bar{I}}\right) \frac{1}{\bar{I}^2}$$

3. Results and discussion

3.1. Optical properties of luminophores

As an oxygen-sensitive phosphor, we chose a Ru(II) diimine complex covalently tethered to the aromatic hydrocarbon pyrene, [(bpy)₂Ru(bpy-pyrene)]²⁺ [32]. This complex exhibits emission from a triplet metal-to-ligand charge transfer state (³MLCT) that is in equilibrium with a non-emissive triplet state on the pyrene; the pyrene triplet state is readily quenched by oxygen. The complex is chemically and thermally stable and it has a significantly larger oxygen sensitivity (>100-fold) and higher two-photon absorption cross-section (37 and 7 GM at 780 and 880 nm respectively) than other complexes, for example, Pt and Pd porphyrins (a few GM [18]; the unit, GM (Göppert-Mayer) represents 10⁻⁵⁰ cm⁴ s [33]). The quantum yield value was measured to be 0.025 for the fully deoxygenated Ru complex. This phosphor was complexed with TEG to enhance hydrophilicity and paired with a reference fluorophore, c540. c540 has an emission profile that is independent of oxygen levels and spectrally separate from Ru-TEG, enabling quantification of the concentration of oxygen as the ratio of intensities of the phosphor and the reference fluorophore (i.e., $\bar{I} = I_{\text{phos}}/I_{\text{fluor}}$). This ratiometric approach eliminates optical artifacts due to, for example, spatial and temporal fluctuations in the intensity of illumination. More importantly, it eliminates the need to know the concentration of the phosphor in the medium and mitigates effects due to light scattering associated with different samples.

The maximum in UV-Vis absorption of [(TEG-bpy)₂Ru(bpy-pyr)]Cl₂ in water was 464 nm with room temperature luminescence in deoxygenated water at 646 nm (Fig. 2a). The luminescence intensity and lifetime were quenched in the presence of oxygen. The emission spectra of Ru-TEG in deoxygenated and aerated water are shown in Fig. 2a. The excited state lifetimes, measured by nanosecond transient absorption spectroscopy, were 6.7 μs in deoxygenated (green) and 1.1 μs in aerated (black) water. These lifetime values as well as an estimated K_{sv}^{τ} value from an independent calibration experiment with Ru-TEG dissolved in water (also see following sections and Supplementary Figure S4) indicate that oxygen sensitivity of Ru-TEG when freely dissolved in water is greater than that of Ru-TEG when incorporated within PUAN nanoparticles. Our observations (i.e., the decrease of oxygen sensitivity) could result from the inaccessibility of oxygen to Ru-TEG incorporated in PUAN.

3.2. Synthesis and size characterization of oxygen-sensing nanoparticles

Amphiphilic reactive oligomers, UAN [(26),(27),(34),(35)] chains are composed of a hydrophilic PEO segment and a hydrophobic PPO segment that are covalently connected *via* urethane linkage (Fig. 1b). On mixing with water, UAN chains form micelle-like nanoparticles *via* self-assembly due to intermolecular hydrophobic interaction (Fig. 1c). Through the formation of micelle-like UAN nanoparticles in water, PEG segments oriented toward the water phase form a hydrophilic shell whereas PPO segments and their vinyl groups form a hydrophobic core [34–36]. The chemical crosslinking polymerization takes place only between vinyl groups in the hydrophobic core within UAN nanoparticles, and allows for a permanent lock-in of their micelle-like nanostructure; this transformation converts UAN nanoparticles to core-crosslinked polymeric nanoparticles (Fig. 1d).

We note that Ru-TEG is water-soluble because it exists as a divalent ion consisting of polar bipyridine and water-miscible TEG. Furthermore, we observed no measurable aggregation of Ru-TEG by dynamic light scattering (DLS) when it was dissolved in water (data not shown); this observation suggests that Ru-TEG does not form micelles in water or that the size of Ru-TEG micelles would be extremely small (<0.6 nm).

Average diameters of Ru-TEG-PUAN and Ru-TEG-c540-PUAN were 41.50 ± 0.799 and 41.53 ± 0.755 nm with polydispersity index (PDI) of 0.120 ± 0.009 and 0.109 ± 0.010 , respectively, as measured by DLS. We confirmed that the size distributions remained almost the same at both 25 and 37 °C (Table 1). This observation suggests that our oxygen-sensing nanoparticles do not exhibit swelling or shrinkage under our typical experimental conditions. We also verified that the luminophores remained stably incorporated within PUAN for several months. The phosphorescence and its oxygen dependence was apparent for a 10 month-old suspension of Ru-TEG-c540-PUAN nanoparticles (Fig. 2b, red and blue lines) whereas no signal was seen from the solvent eluted from a filter column upon passage of this suspension (Fig. 2b, green and yellow lines) or from DI water (Fig. 2b, black line).

3.3. Biocompatibility of oxygen-sensing nanoparticles

Oxygen-sensitive phosphors (typically metal–ligand complexes) can be highly toxic to cells, because they are small amphiphilic molecules that home to cellular lipid bilayers. Furthermore, their interaction with oxygen results in the production of highly energetic singlet oxygen species that are short lived but extremely reactive [37,38]. Ceroni *et al.* have recently shown that PEGylated phosphorescent dendrimers were not phototoxic, demonstrating nanoparticle incorporation as a viable route to phosphor biocompatibility [39]. To evaluate the toxicity of Ru-TEG, we cultured OSCC-3s in 200 μ m-thick alginate discs (4 mm in diameter) in contact with either freely dissolved or nanoparticle-encapsulated phosphor; the disk format allows for the maintenance of a uniform oxygen distribution within the volume seeded with cells [9]. We assumed 100% incorporation of Ru-TEG into the PUAN nanoparticles in calculating an appropriate concentration of free Ru-TEG (i.e., 112.5 nM in culture media) for comparison. We observed that culture of OSCC-3s in media containing free Ru-TEG resulted in significant cell death; both live-dead staining and DNA quantification showed significant cellular death, as early as the first day of culture (also see Supplementary Fig. S4); by day 7, viability was less than 25% viability, even in the absence of exposure to the excitation light (Fig. 3a i–iii and b). The significant cell death with free Ru-TEG indicates that Ru-TEG presents direct toxicity to the cells. In contrast, OSCC-3s cultured within discs with phosphor encapsulated in nanoparticles showed similarly high cell viability compared with those within control discs (i.e., no Ru-TEG-c540 nanoparticles within discs), by both live-dead staining (Fig. 3a iv–ix) and DNA quantification (Fig. 3b) out to 7 days of culture. In addition, OSCC-3s cultured both with and without oxygen-sensing nanoparticles proliferated by a factor of about 1.5–2 over the culture period (Fig. 3b). Interestingly, cells cultured with the nanoparticles exhibited enhanced proliferation as inferred from DNA quantification; however this may be an artifact resulting from some level of either cellular endocytosis, or simply binding of Ru-TEG-c540-PUAN nanoparticles, which would lead to increased green signal measured during the fluorescent DNA assay. Overall, these results demonstrate that encapsulation of phosphors within nanoparticles eliminates the toxicity of these luminophores.

In vivo, we also observed that mice injected with oxygen-sensing nanoparticles (i.e., Ru-TEG-PUAN at imaging concentrations) did not show any immediate (acute) toxicity. Further, we kept them alive for up to a month after the injection with no apparent adverse effect (data not shown). We therefore conclude that encapsulation of Ru-TEG within PUAN nanoparticles is effective in eliminating the toxicity of the free phosphor. The biological

compatibility of PUAN nanoparticles has previously been demonstrated by use of similar particles for delivery of chemotherapeutic agents [36].

3.4. Evaluation of both direct phosphorescence and ratiometric approaches for optically measuring oxygen-depletion

To characterize oxygen imaging based on direct phosphorescence intensity, I_{phos} and normalized intensity, $\bar{I}$ with PUAN nanoparticles, we focus on the depletion of oxygen due to the metabolic activity of tumor cells in 3-D scaffolds. We [((9),(40)), (41)] and others [42,43] have shown that the presence of such gradients influences the pro-angiogenic potential of the cancer cells. Fig. 4 presents the fabrication and geometry of our oxygen-sensing microfluidic scaffold: a 500 μm -thick slab of alginate (4 mm $\times$ 4 mm) seeded with OSCC-3 was sealed within a plexiglass jig such that one edge was adjacent to a single microchannel. Media delivered through the channel was the sole source of oxygen in this system, such that a steady-state oxygen profile could be reached in which diffusion from the channel balanced cellular consumption within the bulk.

The ratio of phosphorescence and fluorescence intensities ($\bar{I} = I_{\text{phos}}/I_{\text{fluor}}$) was calibrated by delivering media maintained at controlled oxygen levels (Fig. 5a and b). Our calibration data for the two cell seeding densities were not statistically different; the coefficient of variation (CV) in percentage, defined as standard deviation divided by mean, ranged from 11 to 17 %. Therefore, we attained a master calibration curve as an average of individual curves for six devices (3 for 5×10^6 cell/mL and 3 for 2×10^7 cell/mL; Fig. 5d), and used this master curve to convert the ratio of intensities, $\bar{I}$ to the concentration of dissolved oxygen, c_{O_2} with $\bar{I}_0/\bar{I} = 1 + K_{\text{SV}}\bar{I}c_{\text{O}_2}$. From the SterneVolmer plot (Fig. 5d), the estimated $K_{\text{SV}}\bar{I}$ (i.e., slope of the black solid line; oxygen sensitivity of Ru-TEG) was $(4.056 \pm 0.55) \times 10^{-3} \mu\text{M}^{-1}$. Note that we estimated propagated uncertainties in both $\bar{I}$ and c_{O_2} to present errors (also see Section 2.16 in Material and Methods).

Wide-field luminescence images were taken using both Ru-TEG and c540 emission channels. The micrographs in Fig. 6a show representative phosphorescence intensity with background subtracted (I_{phos}) as a color map for two different seeding densities, 5×10^6 (left; Fig. 6a i) and 2×10^7 cell/mL (right; Fig. 6a ii), 45 min after the initiation of flow through the channel. The variation of I_{phos} represents the variation in the concentration of oxygen, from the ambient oxygen source (left; red) toward hypoxic bulk (right; blue). For the same two seeding densities, Fig. 6b presents the temporal evolution of z-averaged $I_{\text{phos}}(y)$ and $I_{\text{fluor}}(y)$. The temporal evolution of $I_{\text{phos}}(y)$ reveals that our experimental system approached steady-state after ~ 30 min (Fig. 6b). Fig. 6c presents averaged profiles of phosphorescence (solid lines) with standard deviations (dashed lines) for three experiments at each density of cells. The increase in $I_{\text{phos}}(y)$ as a function of distance from the channel in Fig. 6c indicates the depletion of oxygen within the cell-seeded scaffolds. As expected, the spatial variation of $I_{\text{phos}}(y)$ was more rapid for the higher density of cells. Fig. 6d presents the calibrated intensity, $\bar{I} = I_{\text{phos}}/I_{\text{fluor}}$. The same trends are visible in the variation of $\bar{I}$ as in I_{phos} , but the large relative amplitude of the noise and uncertainty in the reference signal, I_{fluor} led to increased noise and uncertainty in $\bar{I}$ relative to that in I_{phos} .

To proceed to an evaluation of the actual distribution of oxygen within the scaffolds, Fig. 7 presents profiles of oxygen concentration, $c_{\text{O}_2}(y)$ calculated with the Stern–Volmer equation from the profile of phosphorescence in Fig. 6c (Fig. 7a) and from the profile of ratiometric intensity in Fig. 6d (Fig. 7b). In Fig. 7a, we see that the profiles of concentration calculated directly from I_{phos} present unphysical values: far from the source, the calculated concentrations are negative; at the source ($y = 0$), the values for the two densities of cells are

far from one another and from the expected value for ambient oxygen of 273 μM . The negative c_{O_2} resulted predominantly from relatively lower values of $I_{\text{phos},0}$ during the calibration compared with I_{phos} when $y > 1$ mm during the optical measurements with live cells. These inaccuracy and errors may arise from the shift of $I_{\text{phos},0}$ associated with temporal fluctuations of the intensity of our mercury lamp. Cells were unlikely to affect the shift because they were treated with formaldehyde for ~ 1 h prior to the calibration. The I_{phos} -based conversion of c_{O_2} illustrates the liabilities of working with non-ratiometric intensities.

In Fig. 7b, we observe the propagation of the large noise and uncertainty that appeared in the ratiometric intensities. Nonetheless, the average values of $c_{\text{O}_2}(y)$ calculated from $\bar{I}$ are reasonable: far from the source, they tend toward zero and at the source the values for both densities of cells approach the appropriate value for ambient oxygen. To obtain more quantitative information from these profiles, we compare with numerical solutions to a 1-D model of diffusion and reaction of oxygen in the cell-seeded scaffolds with the assumption of Michaelis–Menten kinetics (also see Section 2.13 in Materials and Methods). The solid lines (dark red and dark blue) in Fig. 7b present the calculated profiles in which we have used independently measured values for $V_{m,\text{cell}}$ and adjusted values for K_M . We used a single value of $K_M = 12.3 \mu\text{M}$ for the two cell seeding densities by computational iterations to aim for a square correlation coefficient (R^2) greater than 0.95. The agreement between the model and the data are good - R^2 and root mean square error (RMSE) values between the experimental data and computational calculations [28] were 0.99 and 29 μM for 5×10^6 cell/mL and 0.98 and 34 μM for 2×10^7 cell/mL. Our predicted K_M implies that it is generally appropriate to assume zeroth order consumption kinetics under typical cell culture conditions, because it is only 4.5% of c_{O_2} (i.e., 273 μM), and therefore the assumption of constant oxygen consumption rate is reasonable from Eq. (2). This agreement indicates that, with our ratiometric approach, the Ru-TEG nano-particles can provide quantitative measurements of the spatial distribution of oxygen within a 3-D culture via direct imaging.

We note, though, that these ratiometric measurements are compromised in the case shown by the large noise and uncertainty. In particular, the large values of the propagated uncertainties in the calculated values of c_{O_2} limit precision (averaged deviation of $\sim 74 \mu\text{M}$) with respect to concentration. An important improvement in precision would be achieved by increasing the signal to noise of the fluorescence by, for example, increasing the loading of the reference dye. Another important improvement could be achieved with the use of a more stable light source; temporal and spatial variations of the excitation light increase the uncertainty of the Stern–Volmer parameters.

Oxygen heterogeneity will inevitably impact cellular behavior [9,40], and while our work demonstrates standard Michaelis–Menten-like consumption over a short period of time, more complex temporal and spatial profiles can emerge such that cells proliferate and adapt to different oxygen environments [44–46]. Such behavior would have obvious implications for tumor progression, as temporal variations in hypoxia are thought to be associated with development of a more aggressive and therapy resistant tumor type [47]. Furthermore, as 3-D culture platforms become more ubiquitous, it is becoming more important to have flexible techniques for the measurement of oxygen levels that are often variable throughout the bulk of these systems. Optical measurement with our encapsulated nanoparticle sensors provides one practical solution, enabling online measurement of oxygen level with high spatial as well as temporal resolution.

3.5. In vivo oxygen imaging of mouse vasculature

We also employed our oxygen-sensing nanoparticles for the quantitative measurement of oxygen concentration in mouse vasculature. We chose to image the saphenous vasculature because it was easily accessible and relatively free from artifacts due to breathing motion. We pursued lifetime measurements for *in vivo* oxygen measurements since lifetime is known to be independent of artifacts such as optical scattering and luminophore concentration [48]. Furthermore, *in vivo* imaging requires optical sectioning and thus laser scanning. In this geometry, the ratiometric approach used *in vitro* cannot be used quantitatively because a good proportion of phosphorescent molecules are saturated. In other words, many of them are still in the excited state and unavailable to re-excitation, leading to much slower cycling times. Because phosphors that are sensitive to physiologically relevant concentrations of dissolved oxygen possess lifetimes that are 1000-fold longer than typical fluorophores, the rate of molecular saturation (when the rate of excitation = the rate of de-excitation) is 1000-fold lower than normal. For all practical purposes, molecular saturation cannot be ignored while imaging phosphorescence in any laser scanning configuration [19]. In this case, the signal intensity becomes dependent on excitation intensity, making lifetime measurements necessary for oxygen quantification. To achieve three-dimensionally resolved measurements, we chose to employ two-photon excitation because, relative to single photon, it enables deeper penetration into tissues and effectively reduced intensity in UV bands that can be phototoxic. Lecoq *et al.* have also recently demonstrated the further multi-functionality of two-photon microscopy, using this modality to measure both partial pressure of oxygen and blood flow in deep cerebral vessels [49].

Fig. 8a shows the lifetime-based Stern–Volmer plot for Ru-TEG-PUAN nanoparticles dispersed in water equilibrated at various concentrations of oxygen

($K_{sv}^{\tau} = (5.18 \pm 0.24) \times 10^{-3} \mu\text{M}^{-1}$). Using multi-photon microscopy, lifetime and intensity measurements showed almost the same Stern–Volmer constants when the multiphoton beam was constrained to ensure minimal molecular saturation into the phosphorescence state (low numerical aperture (NA = 0.5) optics with μs -duration pulses; Supplementary Fig. S5). Our intensity-based oxygen sensitivity measured with standard linear microscopy appeared

to be ~27% less ($K_{sv}^I = (4.056 \pm 0.55) \times 10^{-3} \mu\text{M}^{-1}$, Fig. 5d). More generally the specific value for the Stern–Volmer constant varied significantly with specific experimental factors such as the microscope objective NA, the laser intensity, and the polymeric matrix in which the phosphor was encapsulated. Thus it was imperative to calibrate the oxygen sensitivity under the same conditions where the oxygen measurements were performed. Additionally calibration by intensity and lifetime often yielded differing Stern–Volmer constants, a result not expected from ideal dynamic quenching. This spread in measured oxygen sensitivity can be caused by a variety of factors including 1) phosphor saturation [19], 2) micro-heterogeneity in phosphor accessibility to oxygen [24], and 3) the possibility for partial quenching through the phosphor ground state (static quenching).

Molecular saturation is known to be a problem with fluorescence laser scanning measurements in which the beam is focused to locally high intensities, causing a probability for excitation that approaches the probability for de-excitation ($\sim 1/\text{lifetime}$). This problem is expected to be 1000-fold worse in phosphors [19] as compared to fluorophores (with ns-lifetimes). Under standard multi-photon imaging conditions, intensity-based Stern–Volmer constants were dependent on intensity and smaller than the lifetime-based constants (data not shown); they could only be made to match lifetime-derived constants by modulating the beam and using low NA optics. These results suggest that under standard multi-photon imaging conditions, oxygen probes will be saturated, especially under low-oxygen conditions.

The Ru complex is known to be environmentally sensitive [24], so we were not surprised to observe about a three-fold reduction in sensitivity when the Ru-TEG was encapsulated within the PUAN matrix (Supplementary Fig. S6). Local environments around the incorporated Ru-TEG in PUAN may be less accessible to oxygen molecules. In addition, static quenching may have played a role when Ru-TEG was incorporated in PUAN due to weak ground-state interactions between oxygen and aromatic groups in the Ru-TEG-PUAN complex. In this case, the intensity quenching is expected to be nonlinear and greater than the lifetime quenching [23]. Differences in lifetime and intensity quenching have been commonly observed, including a similar reduction of sensitivity using intensity measurements [50].

We used the calibration data (Fig. 8a) to translate phosphorescence lifetime (also see Fig. 8e) into concentration of oxygen. Phosphorescence lifetime values of Ru-TEG in the saphenous artery (red) and vein (blue), estimated from the single-exponential fit (solid curves in Fig. 8e), were 4.73 and 13.4 μs for artery and vein, respectively. The concentration of oxygen in the artery (413 μM) appeared to be higher than the ambient oxygen level (273 μM); this higher value arose due to our use of pure oxygen as the anesthesia carrier gas. The concentration in the vein was significantly lower (21 μM), as expected. Fig. 8f shows the arterial measurement as the partial pressure of oxygen (p_{O_2}) in the anesthesia carrier gas was repeatedly varied over the course of 1 h (% oxygen levels in the gas are reported at the top of the graph); the concentration of oxygen in the artery varies by a factor of >3 . Note also that the plasma oxygen concentration at which hemoglobin (Hb) is half-bound is 37.5 μM ($\sim 3\% p_{\text{O}_2}$) [51]. Thus, Hb exhibits a significantly lower buffering effect from 100 to 21 % p_{O_2} as compared to 21 to 10 % p_{O_2} . Using this method, plasma measurements are highly reproducible and repeatable. Note that the high oxygen levels correspond to short lifetimes which are at the temporal limit of our Pockel's cell laser modulator (~ 0.5 ms). Thus these data points exhibit more noise than those at lower oxygen levels.

Our *in vivo* measurements demonstrate that these nanoparticles can be used to provide reproducible, three-dimensionally resolved oxygen measurements at capillary spatial scales. Our measurements further highlight some of the challenges inherent to any phosphor-based oxygen quantification *in vivo*. These results imply important operation criteria, including the necessity for appropriate calibration for a particular operating environment, as well as operation within a range of excitation parameters to avoid variation in the Stern–Volmer constant. Based on their small size, the nanoparticles we present also could provide the ability for tissue oxygen imaging in biological systems with leaky vasculature, such as tumors or tissues where extensive matrix remodeling occurs. Direct injection of nanoparticles, local Mannitol administration [52] for temporarily permeabilizing vasculature, or functionalization of the PUAN nanoparticles with homing peptides could provide alternative avenues of delivery to tissues of interest. Potential future advantages could include tunability of size, hydrophilic/hydrophobic balance, and functionality of nanoparticles via changes in PEG or PPO chain length or chemical modifications of UAN chains. Furthermore, the potential tunability of these oxygen sensors could lead to the development of multi-purpose nanoparticles, for instance, for oxygen-pH sensing.

4. Conclusions

We have presented a new set of tools to quantify the concentration of dissolved oxygen for applications both *in vitro* and *in vivo*, by encapsulating a specially designed Ru-based phosphor within PUAN nanoparticles. The phosphor represents an improvement over widely used chemistries with respect to sensitivity to oxygen concentration ($5.2 \times 10^{-3} \mu\text{M}^{-1}$) and absorption cross-section for two-photon excitation (37 GM at 780 nm). The UAN amphiphilic reactive oligomer offers a number of advantages as a nano-vehicle over

amphiphilic block copolymers, surfactant micelles, and dendrimers, including: 1) simple self-assembly driven synthesis, 2) high stability and long term incorporation of phosphors due to chemical crosslinking of hydrophobic cores. We have used these nanoparticles to demonstrate non-invasive and real-time measurements of oxygen gradients occurring due to cellular consumption in a 3-D cell culture platform, as well as *in vivo* oxygen levels in mouse vasculature. We anticipate that the versatility of the materials and techniques we present will lead to the adoption of similar approaches for online oxygen measurement in a range of experimental systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Schematic illustration representing synthesis of oxygen-sensing nanoparticles. a. Chemical structure of oxygen-sensitive phosphor, $[(\text{TEG-bpy})_2\text{Ru}(\text{bpy-pyr})]\text{Cl}_2$ (Ru-TEG). b. Amphoteric reactive oligomer, urethane acrylate nonionomer (UAN) consisting of hydrophilic polyethylene oxide segment (PEO; blue) and hydrophobic polypropylene oxide segment (PPO, gray) with reactive vinyl groups (black). c. UAN nanoparticle loaded with Ru-TEG (red circle). d. Ru-TEG-PUAN nanoparticle synthesized via chemical cross-linking polymerization. e. Ru-TEG-PUAN nanoparticle with a post-incorporated reference fluorophore, coumarin540 (c540; green circle). f. Schematic representation of principle of oxygen imaging. Purple arrows represent incident excitation light. Red arrow represents phosphorescence emission from Ru-TEG in the presence (left) and absence (right) of oxygen molecules. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Photophysical properties of free Ru-TEG and Ru-TEG encapsulated in nanoparticles (Ru-TEG-c540-PUAN). a. Emission spectra of Ru-TEG in deoxygenated (green) and aerated (black) water. b. Emission spectra of DI water (black), spin-through liquid at ambient oxygen level (green), deoxygenated spin-through liquid (yellow), re-dialyzed 0.25 % [w/v] Ru-TEG-c540-PUAN at ambient oxygen level (red), and deoxygenated Ru-TEG-c540-PUAN (blue). The adsorption spectra of spin-through liquids and the DI water are indistinguishable. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Biocompatibility of oxygen-sensing nanoparticles. a. Fluorescence micrographs showing cells stained by 5 μ M calcein-AM (green – live) and 5 μ M ethidium homodimer-1 (red – dead) after 7 day of culture of OSCC-3s seeded in 4% [w/v] calcium alginate discs (200 mm-thick). b. DNA content per alginate disc, normalized to day 1-DNA amount without exposure. Images and grouped bar graphs from left to right correspond to: free Ru-TEG dissolved in culture media, no oxygen-sensing nanoparticles dispersed in OSCC-3-seeded alginate discs, and Ru-TEG-c540-PUAN nanoparticles dispersed in alginate discs. Images from top to bottom and bar graphs within each group from left to right represent no exposure to excitation light (392 nm), 10 s-exposure, and 30 s-exposure per day. Bar graphs with black squares inside represent normalized DNA content on day 7. Error bars represent standard deviation of mean. Statistical significance *** represents $p < 0.001$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4.

Fabrication of 1-channel microfluidic system for visualization and optical measurement of oxygen-depletion lengths (Krogh lengths). a. Schematic diagram showing the fabrication of microfluidic system. The red line indicates polyethyleneimine (PEI) coating. b. Schematic representation of oxygen-sensing microfluidic scaffold seeded with cells (top-down view). Dispersed cells are depicted as double circles and oxygen-sensing nanoparticles as red circles. The pink shading represents steady-state distribution of oxygen within the cell-seeded scaffold. W is width of the scaffold. The Krogh length, λ_K , is the characteristic distance over which oxygen diffuses into the scaffold from the microchannel. c_0 is oxygen concentration at the microchannel-scaffold interface. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 5.

Intensity-based calibration of oxygen-sensing nanoparticles. a-b. Representative transient profiles of luminescence intensity during intensity-based calibration. Frame (a) presents temporally varying phosphorescence (Ru-TEG; red) and reference fluorescence (coumarin540; green) intensity as oxygen concentration was incrementally decreased from ambient (0 min) to deoxygenated (120 min) states (a). Frame (b) presents the ratio of phosphorescence and fluorescence intensities (i.e., $\bar{I} = I_{\text{phos}}/I_{\text{fluor}}$) calculated from the data presented in (a). The insets present color-mapped micrographs of $\bar{I}$ from an OSCC-3-seeded scaffold (2×10^7 cell/mL) equilibrated at various concentrations of oxygen. c and d. Stern–Volmer plots obtained from the intensity-based calibration with cell-seeded scaffolds: 3 scaffolds at 5×10^6 cell/mL and 3 scaffolds at 2×10^7 cell/mL. Circles are the mean values of I_{phos} (c; red) and $\bar{I}_0/\bar{I}$ (d; black), and error bars are uncertainties. The solid lines are linear regressions weighted by uncertainties (standard deviation of residuals, $S_{y,x} = 0.1568$ (c) and 0.1011 (d)), and gray dotted lines represent 95% confidence interval for the linear regressions. The slopes of the linear regressions are $K_{SV}^{\bar{I}_{\text{phos}}} = (3.8 \pm 0.37) \times 10^{-3} \mu\text{M}^{-1}$ (c) and $K_{SV}^{\tau} = (4.06 \pm 0.55) \times 10^{-3} \mu\text{M}^{-1}$ (d). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 6.

Visualization and quantification of oxygen-depletion in microfluidic tumor model. a. Phosphorescence micrographs of alginate scaffolds seeded with Ru-TEG nanoparticles and OSCC-3 cells at 5×10^6 cell/mL (frame i) and 2×10^7 cell/mL (frame ii). The color-maps show phosphorescence intensity from which the background intensity was subtracted pixel-by-pixel (also see Section 2.11 in Materials and Methods). Red and blue represent ambient and hypoxic oxygen levels respectively. b. Transient profiles along y -direction of images as in (a). Red lines represent phosphorescence from Ru-TEG with background subtracted (I_{phos}); green lines represent fluorescence of the Coumarin 540 reference (I_{fluor}). The profiles were extracted from frames taken every minute out to 45 min c and d. Profiles of I_{phos} (c) and $\bar{I} = I_{\text{phos}} / I_{\text{fluor}}$ (d) along y -direction at 45 min for 5×10^6 cell/mL (red) and 2×10^7 cell/mL (blue). Dash lines represent standard deviation of I_{phos} or I from three replicates for each cell seeding density. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 7.

Distribution of oxygen concentration within the microfluidic tumor model. Spatially resolved distributions of oxygen concentration calculated from intensity data in Fig. 6. a. Concentration profiles calculated from phosphorescence intensity (Fig. 6c). b. Concentration profiles calculated from ratiometric intensity (Fig. 6d). In (a) and (b), red and blue represent cell seeding densities of 5×10^6 cell/mL and 2×10^7 cell/mL, respectively. Solid and dashed lines represent $c_{O_2}(y)$ and estimated uncertainty respectively, from three replicates for each cell seeding density. Dark red and dark blue solid lines in (b) are computationally calculated oxygen concentration in a finite element model with an assumption of Michaelis-Menten kinetics: $D_{O_2} = 2.76 \times 10^{-9} m/s$, $c_0 = 273 \mu M$, $V_{m, cell} = 5.4 \times 10^{-17} mol/s \cdot cell$, $K_M = 12.3 \mu M$, $\rho_{cell} = 5 \times 10^6 cell/mL$ (dark red) and $2 \times 10^7 cell/mL$ (dark blue). In these calculations, concentration boundary conditions were maintained at the channel walls ($c_{y=0} = c_0$), and no-flux conditions were maintained at other boundaries. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 8.

Lifetime-based quantification of oxygen concentration in vivo. a. Stern-Volmer plot based on phosphorescence lifetime of for Ru-TEG-PUAN nanoparticles dispersed in water. Black squares are the mean values of τ_0/τ , and error bars are standard deviation (multiple measurements at each c_{O_2} were used from two independent calibration experiments). The solid line is a linear regression (standard deviation of residuals, $S_{y,x} = 0.1622$), and gray dotted lines represent 95% confidence interval for the linear regression. b. Schematic of *in vivo* oxygen imaging platform. c. Mouse saphenous vasculature, exposed by an incision through the skin, leaving the fascia intact. d. Multiphoton image of saphenous vessels from the region of interest highlighted in (c; white square) after intracardiac injection of oxygen-sensing nanoparticles. e. Phosphorescence lifetime decay of Ru-TEG-PUAN nanoparticles in saphenous artery (red dots) and vein (blue dots). Solid lines in red and blue are single-exponential decay curves fit to the experimental data points. f. Oxygen concentration in mouse artery while varying the oxygen partial pressure in anesthesia carrier gas (percentages are indicated by grayscale bars). Lifetimes were measured at the white filled square in (d) and converted into oxygen concentrations with the Stern-Volmer parameters extracted from the plot in (a) and the Stern-Volmer equation (Eq. (1)). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1

Size distributions of oxygen-sensing nanoparticles measured by Dynamic Light Scattering (DLS) at 25 and 37 °C.

	Temperature [°C]	Average diameter (mean $\pm$ std. dev.) [nm]	Polydispersity index (mean $\pm$ std. dev.)
1% [w/v]	25	41.53 $\pm$ 0.9717	0.119 $\pm$ 0.010
Ru-TEG-PUAN	37	41.46 $\pm$ 0.6756	0.120 $\pm$ 0.009
0.5% [w/v]	25	41.11 $\pm$ 0.4266	0.120 $\pm$ 0.009
Ru-TEG-c540-PUAN	37	41.39 $\pm$ 1.012	0.106 $\pm$ 0.013