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Two-photon microscopy of oxygen: polymersomes as probe carrier vehicles

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Abstract

Oxygen concentration distributions in biological systems can be imaged by the phosphorescence quenching method in combination with two-photon laser scanning microscopy. In this paper we identified the excitation regime in which the signal of a two-photon-enhanced phosphorescent probe¹ is dependent quadratically on the excitation power (quadratic regime), and performed simulations that relate the photophysical properties of the probe to the imaging resolution. Further, we characterized polymersomes as a method of probe encapsulation and delivery. Photo-physical and oxygen sensing properties of the probe were found unchanged when the probe is encapsulated in polymersomes. Polymersomes were found capable of sustaining high probe concentrations, thereby serving to improve the signal-to-noise ratios for oxygen detection compared to the previously employed probe delivery methods. Imaging of polymersomes loaded with the probe was used as a test-bed for a new method.

Keywords

phosphorescence; oxygen; two-photon microscopy; polymersome; dendrimer; porphyrin

Introduction

The importance of oxygen measurements in biological systems cannot be overestimated, since oxygen is a key component of biological energy metabolism. The ability to image oxygenation with high spatial and temporal resolution is, therefore, a very appealing objective. A number of methods for quantification of oxygen in biological environments have been proposed in the past,^{2–6} but none of them so far provides means for high-resolution microscopy with three-dimensional (3D) capability. We are developing such a technology by combining measurements of the quenching of phosphorescence by oxygen⁷ with two-photon laser scanning microscopy.⁸ Previously, we have reported two-photon-enhanced phosphorescent nanoprobes^{9–11} and demonstrated their performance in pilot imaging experiments.¹ One of the key issues with respect to the cellular imaging application is related to the probe delivery into cells. In this paper we explore polymersomes¹² - self-

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Supporting Information. Detailed information about the polymersome synthesis and characterization, sample preparation, design of the two-photon phosphorescence imaging system and numerical simulations. The structure of the probe (scheme S1), phase contrast micrographs of polymersome (Fig. S1) and SNR characteristics of the two photon system (Fig. S2).

assembled soft polymeric vesicles - as probe carrier vehicles. Simultaneously, we address questions of excitation volume and imaging resolution in oxygen microscopy by two-photon-excited phosphorescence - both closely related to the photophysical properties of the probe.

Among existing oxygen measurement techniques, phosphorescence quenching is attractive because of its ability to perform fast, absolute measurements of pO_2 (partial pressure of oxygen), independent of the optical properties (e.g. absorption and scattering) of measurement environments. There are numerous examples of the application of phosphorescence quenching in the microscopy and imaging of biological systems.^{13–17}

Two-photon excitation offers several advantages over linear methods. First, two-photon excitation makes it possible to perform optical sectioning without loss of photons to a confocal pinhole,¹⁸ which is required in the case of linear excitation to eliminate out-of-focus signals. Secondly, two-photon excitation is usually performed in the near-infrared (NIR) region,^{19,20} where absorption by endogenous chromophores is at a minimum,²¹ allowing for deeper penetration of the excitation light²² and inflicting less photo-damage.⁸ Furthermore, due to the highly localized nature of the two-photon energy distribution, photochemical damage inflicted by bleaching of the probe and various quenching reactions can be confined to near the focal volume⁸ - a feature especially important for phosphorescence lifetime imaging.

Two-photon excitation has special appeal for intracellular oxygen measurements,^{23–26} where maximal resolution and low photodamage are particularly stringent requirements.^{27,28} In order to conduct such measurements, a phosphorescent probe must be delivered into a cell, and the imaging then needs to be performed with the highest possible spatial resolution, which demands the minimal attainable excitation volume.

In the initial two-photon oxygen imaging experiments,¹ extremely small amounts of the probe could be delivered into cells. The probe was dissolved in a cell-growth medium and co-internalized together with surface-modified latex microspheres *via* receptor-mediated endocytosis. As a result, the probe was found only in thin solution layers surrounding the microspheres in the endosomal compartments. It is obvious that much larger amounts of the probe would be delivered into cells if carrier-vesicles would have their entire internal volume filled with a probe solution. A variety of nano-carriers have been used for emissive sensors, including solid nanoparticles (sol-gel^{26,29}, silica³⁰, polystyrene^{31–33}, lipobeads³⁴) and membrane carriers encapsulating liquid solutions of various luminescent probes (liposomes^{35–37} or carriers built using layer-by-layer self assembly of polyelectrolytes^{38,39}). The primary role of these carriers was to protect sensor molecules from bio-fouling and non-specific quenching inside the cell.

In this paper we evaluated polymersomes¹² as carrier vehicles for two-photon phosphorescent probes. Polymersomes are bilayer vesicles assembled from block-copolymers, which can be varied to fine-tune membrane strength^{40,41} and responsiveness,⁴² thus overcoming the fragility issues inherent in liposome delivery systems.^{34,43} Polymersomes can be decorated with cell-penetrating peptides to enhance delivery in to the cell,⁴⁴ or decorated with other motifs to encourage binding to specific targets.⁴⁵ When using polymersome-encapsulated probes for oxygen measurements it is important that there are no interactions between the carrier and the probe, and that the response of the encapsulated probe to oxygen is not different from that of the probe in bulk solution. In other words, polymersomes must not alter the probe's calibration constants and/or impede oxygen exchange between the solution inside the vesicle and the bulk phase.

The second goal was to determine the optimal excitation conditions for high-resolution oxygen imaging and perform imaging experiments in this regime using polymersomes as a model system. The maximal resolution in two-photon microscopy is achieved when the excitation is carried out in the quadratic power regime,⁴⁶ that is within the power range when the observed signal (emission) has quadratic dependence on the incident laser power, as is expected for a two-photon process. At higher powers, deviations from quadratic behavior can be seen either due to higher order effects (three photon, etc.)⁴⁷ or due to saturation. In saturation, a near unity fraction of molecules in the immediate vicinity of the laser focus exist in the excited state. At that point, molecules further away from the focus begin to dominate the power dependence within the system. As a result, the total excitation volume becomes enlarged and the resolution decreases.⁴⁶ This effect was first demonstrated for quantum dots, whose two-photon absorption cross-sections are orders of magnitude higher than those of organic dyes. Simulations were performed to determine the dependence of excitation volume on incident power.⁴⁶

Saturation becomes especially problematic in the case of phosphorescent probes when they are excited by high repetition rate laser pulses. Phosphorescence lifetimes are on the order of microseconds or milliseconds, while the pulse repetition rates of Ti:Sapphire oscillators, typically used for multiphoton imaging, are near 10^8 Hz, corresponding to pulse spacing of only ~ 10 ns. As a result, triplet states do not have enough time to decay back to the ground state, and subsequent pulses lead to a rapid build-up of the triplet population. Previous simulations of phosphorescence saturation¹ did not take into account increase in the excitation volume and thus did not provide insight into how saturation changes the imaging resolution. The simulations presented in ref. [46] rely on the assumption that the lifetime of emissive state is shorter than the laser pulse spacing, and therefore are not applicable to phosphorescent probes.

In this paper we first determined the quadratic power range within which the excitation volume in two-photon phosphorescence lifetime microscopy approaches the diffraction limit. We characterized polymersomes as carrier vehicles for a two-photon-enhanced phosphorescent probe PtP-C343¹ and performed two-photon imaging of polymersomes loaded with the probe in order to demonstrate responsiveness of this system to changes in oxygenation. The performed imaging experiments simultaneously provided evidence for 3D capability of the method. Further, we carried out simulations in order to determine how the excitation volume depends on the degree of saturation, which is in turn dependent on the excitation power. These estimations allowed us to determine the trade-off between signal and spatial resolution.

Experimental

Optical measurements and imaging

Linear (one-photon) photophysical measurements were performed using standard instrumentation as described in our earlier publications.⁴⁸ Phosphorescence lifetime measurements and oxygen titrations were performed using the systems described previously.⁴⁸ Phosphorescence measurements under two-photon excitation were performed using a modification of the system previously described.¹ For additional details, see Supporting Information.

Raw data processing (both for single kinetic measurements and for imaging) was performed using programs home-written in C and Matlab® (R2008b, Mathworks, Natick, MA). The results were visualized either using Matlab or in ImageJ⁴⁹ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997–

2009). Phosphorescence decays were analyzed by non-linear least-squares using the fitting function in the form:

$$I(t) = I_0 \exp(-t/\tau) + b, \quad (1)$$

and assuming the Poisson counting statistics: $\chi^2 = \frac{1}{N} \sum_i^N (I(t_i) - y_i)^2 \frac{1}{y_i}$. In Eq. 1, I_0 is the initial intensity, τ is the phosphorescence lifetime, and b is the baseline term. Term b accounts for dark counts of the detector, as well as laser scatter and residual fluorescence due to the leak of the excitation during the 'off' state of the Pockels cell. Signal-to-noise ratio (SNR) for kinetic traces was defined as:

$$\text{SNR} = \frac{\sum_i (I(t_i) - b)}{\sqrt{\sum_i (I(t_i) - y_i)^2}} \quad (2)$$

where i is the bin number, t_i is the time corresponding to bin i , y_i is the observed counts in bin i . The numerator in Eq. 2 (signal) includes only the phosphorescence photons. The first bin ($i=1$) was always excluded from the analysis, as it contained fluorescence, laser scatter from the excitation pulse and rise of the phosphorescence from the energy transfer process during the excitation gate.

Images of phosphorescence lifetime (τ), initial intensity (I_0), baseline (b) and SNR were calculated by the analysis program. In addition, images of total intensity (I_{tot}), experimental integrated phosphorescence intensity (I_{phos}) and integrated phosphorescence intensity, as determined by the fit parameters (I_{calc}), were also computed. The definitions are as follows: I_{tot} - total number of photons collected by the detector, excluding photons in the first bin;

$I_{\text{phos}} = \sum_i (y_i - b)$ - total number of experimentally observed phosphorescent photons, first bin excluded, b determined from the best fit; $I_{\text{calc}} = \sum_i (I(t_i) - b) = \sum_i (I_0 \exp(-t_i/\tau))$ - total integrated phosphorescence intensity, as calculated from the best fit. Note, that

$\int_0^\infty I_0 \exp(-t_i/\tau) dt = I_0 \tau$. Therefore, for homogeneously distributed phosphorescence lifetimes τ , integrated intensity images I_{calc} and I_{phos} , as well as image I_0 , reflect the distribution of the phosphorescent probe.

For power studies, a probe solution in water was placed into a 'micro-cuvette', consisting of a polystyrene cloning cylinder (Fisher Scientific) glued to a # 1.5 coverslip. The sample was mounted on the microscope stage. The average laser power (P_{av}) was measured before the Pockels cell with a thermopile power meter (FieldMax II, Coherent). The laser duty cycle (D) was defined as $D = T_{\text{on}} / (T_{\text{on}} + T_{\text{off}})$, where T_{on} and T_{off} are the 'on' and 'off' periods of the Pockels cell per cycle, typically 2 μs and 498 μs respectively. The duty cycle was generally 0.004. The effective power (p) at the sample with the Pockels cell 'on' was calculated based on the laser duty cycle (D), taking into account the transmission efficiency (ϕ_{trans}) of the microscope optics: $p = P_{\text{av}} \times D \times \phi_{\text{trans}}$. The transmission efficiency was defined as the power measured at the sample vs power measured before the Pockels cell with the Pockels cell 'off' and in the full transmission mode. At 840 nm, the transmission efficiency was found to be 1.2%.

Power studies were generally conducted multiple times. Power was varied from high to low, and after the study was completed at least one high power measurement was repeated to

make sure no changes had occurred over the time course of the experiment, which could be up to several hours in duration. The data were plotted as $\log(I_{\text{phos}})$ vs. $\log(p)$ and fit to a straight line in order to determine the quadratic region (slope=2.0).

Numerical simulations

The simulations consisted of two steps: 1) calculation of the photon density distribution in the vicinity of the laser focus; and 2) calculation of the distribution of triplet state molecules based on the kinetics of the photophysical processes occurring in the probe molecule (see Results and Discussion). The calculation of the energy density distribution was based on the Wolf-Richards diffraction theory,^{51,52} in general following ref. [53]. The calculation was performed for the case of light polarized linearly along the X axis. Integration over θ ($0 < \theta < \alpha$) was performed using discretization in 300 steps. For additional details, see Supporting Information.

Results and discussion

Principles of oxygen measurement by phosphorescence quenching

Unlike conventional fluorophores, phosphorescent probes have low fluorescence (emission from the singlet state) and high phosphorescence (emission from the triplet state). The long-lived triplet excited state can be deactivated by emission of a photon, by non-radiative intersystem crossing, or by various quenching processes, including collisional quenching by oxygen.⁵⁴ Phosphorescent probe molecules serve as molecular sensors for oxygen.⁷ The dependence of the phosphorescence lifetime on oxygen concentration is described by the Stern-Volmer relationship:

$$\frac{\tau_0}{\tau} = 1 + k_q \tau_0 [O_2] \quad (3)$$

where τ_0 is the phosphorescence lifetime in the absence of oxygen; k_q is the quenching constant, and τ is the lifetime at oxygen concentration $[O_2]$. The lifetime τ is independent of the probe concentration and can be used for measurements of oxygen in heterogeneous environments.

Two-photon enhanced phosphorescent probe

The design of two-photon-enhanced phosphorescent oxygen probes has been described previously.^{1,9,10,55} Briefly, the probe used in this study, PtP-C343, consists of a phosphorescent (oxygen sensing) platinum porphyrin (PtP), encapsulated within arylglycine (AG) dendrimer (Scheme S1).

Two-photon absorbing antenna chromophores, coumarin-343 (C343), are attached covalently to several peripheral carboxyl groups on the dendrimer, while the remaining carboxyl groups are modified with polyethyleneglycol (PEG) residues. The dendritic shell modulates oxygen diffusion to the phosphorescent core, permitting control over the quenching constant k_q (Eq. 3), thus defining the dynamic range of the sensor. The PEG-residues ensure aqueous solubility of the probe and prevent its interactions with biological macromolecules. The synthesis of the probe followed the earlier described protocol¹ with one exception. Instead of using monomethoxypolyethyleneglycol (Av. MW 750), in this work the probe was peripherally modified with monomethoxypolyethyleneglycol amine (Av. MW 2000). From the synthetic point of view, PEG-amines are more reactive than regular PEG's, which resulted in higher conversion yield per carboxyl group on the dendrimer. More importantly, longer PEG groups reduced aggregation of the probe in aqueous solutions.

The absorption and emission spectra of the probe are shown in Fig. 1A. The absorption spectrum of the probe is a superposition of the spectra of the constituent parts (C343: S_1 460 nm; PtP: S_1 510 nm, S_2 410 nm), indicating that the antenna and the core are not strongly electronically coupled. The steady state emission spectrum shows both C343 fluorescence and Pt porphyrin phosphorescence; however, most of the C343 fluorescence is quenched due to the energy transfer to the porphyrin. The residual C343 emission may be increased at higher excitation intensities if multiple coumarin units are excited, and only one C343 is able to transfer energy to the porphyrin, while the remaining units may dissipate their energy through fluorescence.

As shown previously, phosphorescence of PtP-C343 decays non-single exponentially.¹ Consequently, the classic Stern-Volmer plot of the probe ($1/\tau$ vs pO_2) is non-linear; and for the measurement purposes it is more convenient to use the calibration curve directly relating pO_2 to the lifetime of phosphorescence (Fig. 1B). This curve can be fit to an arbitrary function and used for direct conversion between τ and pO_2 .

It should be mentioned that proper modeling of the complex decay of PtP-C343 would in principle require complete phosphorescence lifetime distribution analysis, such as that based on the maximum entropy method (MEM).⁵⁶ However, for the purpose of oxygen measurements it is appropriate to use "apparent" lifetimes, which are the weighted averages derived from underlying lifetime distributions. Averaging can be done, for example, by fitting a non-single-exponential decay using two-, three- or higher exponential models and deriving the intensity-weighted average lifetimes. Alternatively, one can use single-exponential fitting, which would generate poorer residuals, but would be more robust due to the fewer number of fitting parameters. Single-exponential fitting in this regard can be considered an alternative form of averaging.

The two-photon properties of the probe were measured using the microscope imaging system. The probe was excited by short trains of high repetition rate femtosecond pulses, and the signal was recorded as integrated phosphorescence photon counts (I_{phos}) vs effective laser power on the sample (p) (see Experimental section for details). The probe power plot (Fig. 2) demonstrates that the quadratic regime for the phosphorescence emission can be accessed only at very low powers. The quadratic dependence could be obtained using both bulk solutions of the probe (Fig. 2A) and solutions of the probe inside polymersomes (Fig. 2B). The highest spatial resolution in imaging can be achieved when excitation occurs within the quadratic range of the power curve (*vide infra*). Our previous work was not performed in the quadratic regime.

Characterization of polymersomes

Polymersomes were made from biotin-functionalized polyethylene oxide-block-polybutadiene (biotin-PEO-bBD). This biocompatible polymer easily assembles into vesicles, is liquid at room temperature (facilitating easy sizing of vesicles), and has been characterized and used extensively for a variety of applications.^{40,41,44,45,50,57} The method of thin film rehydration used in this work (65°C, 24 hr in the presence of buffer containing the probe at the desired concentration) results in a polydisperse distribution of giant vesicles, in both size (majority of vesicles >1 μm diameter) and morphology, as shown by the Differential Interference Contrast (DIC) microscopy (Fig. S2). While not ideal for *in vivo* or cell-based experiments, giant vesicles are good for confocal microscopy experiments because they are easily identified using magnification as small as 10 $\times$ and have large aqueous lumens which facilitate a high capacity of probe. The biotin attached to the vesicle membranes allows the vesicles to tightly bind avidin physisorbed to the cover slide substrates. This modification was necessary because small motions occurring during image acquisition (i.e. movements of the scanning stage, diffusion) disturbed the untethered

vesicles, making it impossible to collect reliable spectroscopic data for quantitative analysis. Additionally, tethering of the vesicles to the substrates allowed buffer to be aspirated and enzymes (used to deplete the buffer of dissolved oxygen) to be added to the samples without disturbing vesicles of interest.

Laser scanning confocal microscopy with linear excitation, performed on polymersomes not adhered to glass surfaces, revealed that the probe was contained in vesicles of various morphologies (Fig. 3). The probe presence in the vesicles, as opposed to associated with the membrane, was later confirmed by two-photon measurements (see below).

The current method of polymersome loading involves prolonged heating (see SI), which appeared to slightly alter the photophysical properties of the probe relative to non-treated probe. Thus, for comparisons we used bulk probe subjected to exactly the same heat treatment as the reporter probe encapsulated in polymersomes. The oxygen quenching plots of the heat treated probe in bulk solution and inside polymersomes are compared in Fig. 4, showing that the curves are identical within the measurement error. This result suggests that polymersomes do not impose significant barriers for oxygen diffusion, allowing for rapid exchange of oxygen across the polymersome membranes. Thus, probe-loaded polymersomes are suitable for sampling of intracellular oxygen, while preventing direct contacts between the probe molecules and components of intracellular environment. In addition, polymersomal confinement makes it possible to achieve very high local probe concentrations, thereby increasing the signal strength and improving measurement accuracy. The invariance of the probe's response to the presence of protein (albumin), pH (6.0–8.5), and probe concentration (0.9–50 μM) has been shown previously.¹

While polymersomes have been used with a variety of cargos, there has been a report of unusual photo-activity involving some encapsulated dyes.⁵⁰ A photoactive molecule, a *meso-to-meso* ethyne-bridged bis[(porphinato)Zn], was loaded in the membrane of the polymersome, while a protein was used as the cargo. Upon excitation of the fluorophore, dramatic morphology changes occurred, which in many cases led to complete destruction of the polymersomes. It was postulated that the protein cargo associated with the inner side of the membrane, causing its rigidification. Upon absorption of the light, the membrane was locally heated, and the inner, protein-associated, membrane was unable to expand, thus leading to destruction of the vesicle. In view of this finding it was important to verify that polymersomes loaded with PtP-C343 were stable under two-photon irradiation. Indeed, morphological changes were not observed upon irradiation, suggesting that probe molecules are intraluminal solutes that do not interact with the polymersome membranes.

Two-photon imaging

Two-photon images were obtained by raster scanning the sample using a piezo-stage and collecting phosphorescence decays at each pixel. Piezo scanning is generally slower, but more positionally precise than scanning the laser beam by galvo-mirrors. In the case of fluorescent chromophores, pixel dwell times required to achieve appropriate signal counts are on the order of microseconds. Therefore, fast scanning by galvo-mirrors, widely used in commercial confocal and two-photon microscopes, allows in some cases even for video-rate imaging. However, in phosphorescence lifetime imaging pixel dwell times are on the order of several milliseconds; therefore, fast scanning offers little advantage. On the other hand, increasing the dwell time per pixel improves the kinetic trace and the precision of the lifetime measurement (Fig. S2).

The measurements (Fig. S2) were performed using the power just outside the quadratic regime ($p=9.6 \mu\text{W}$). Under these conditions and probe concentration of $\sim 150 \mu\text{M}$, the total number of collected counts per decay was ca 5–10. Therefore, large numbers of runs

(>1,000) were required to achieve sufficient accuracy in lifetime measurements ($\delta\tau \pm 2-3 \mu\text{s}$). In all cases, 10-fold increase in SNR over a 100-fold increase in the number of runs (N) was obtained, consistent with $\text{SNR} \propto N^{0.5}$. The dependence of the average lifetime on the number of runs shows an increase at low SNR values (Fig. S2A). Considering non-single-exponential nature of the decay, this effect can be explained by the truncation of the decay tail (longer lifetime components) at low SNRs, resulting in shifting the apparent lifetime distribution towards the shorter lifetimes.

Examples of images of a polymersome loaded with PtP-C343 and attached to the glass surface are shown in Fig. 5. The first row contains several intensity maps. In panels A and B images of total integrated intensity (I_{tot}) and total integrated phosphorescence intensity (I_{phos}) are shown. In our experimental system, even in the 'off' state, the Pockels cell leaks some light. While this intensity is generally 100-fold less than the intensity transmitted in the "on" state, it does lead to a constant offset in the intensity maps due to the laser scatter and residual fluorescence from C343 fragments in the probe molecule. This offset can easily be determined as a value of the baseline b from fitting the kinetic data (Fig. 5F). The remaining signal (I_{phos}) after subtraction of the baseline should arise only from phosphorescence.

Images I_{phos} , I_{calc} (Fig. 5C), determined from the best fit parameters I_0 and τ ($I_{\text{calc}} = \int_0^\infty I_0 \exp(-t/\tau) dt = I_0 \tau$), and image I_{tot} are almost identical, indicating that the method of processing accurately reproduces object features as they appear in the traditional total intensity map (Fig. 5A).

The lifetime (D) and oxygen (E) maps are essentially binary images, as expected for a homogeneously oxygenated sample. Various additional fitting parameters are shown in panels F–I. The map of the phosphorescence initial intensity (Fig. 5F) I_0 is completely uniform as expected for homogeneously loaded vesicles. (Assuming equal excitation efficiency, initial intensity of emission I_0 is proportional to the probe concentration.) In heterogeneous samples, e.g. in cells, probe loading can be non-homogeneous, making intensity-based measurements ambiguous. Higher intensity could mean more probe loading rather than lower oxygen concentration. While ratiometric probes, in which a fluorescent non-oxygen sensitive emitter is co-loaded or covalently attached to a phosphorescent probe to serve as a reference, have been proposed to overcome this limitation,^{26,33,58–60} variations in reference signal intensity create an intrinsic source of error in ratiometric imaging method (see for example [1]). For example, the image of baseline b (Fig. 5G), which is a significant part of the total intensity I_{tot} (Fig. 5A), is highly heterogeneous since it arises from scatter (of both signal and laser) and fluorescence and is expected to be wavelength dependent. Baseline b can also depend on re-absorption of the emitted light and autofluorescence from the sample. All these factors are wavelength and location dependent, leading to variations in the total intensity,¹ which cannot be easily predicted and/or determined experimentally.

In contrast, lifetime based measurements are insensitive to probe loading and distribution, thus avoiding the problems of the ratiometric approach. As long as sufficient numbers of photons are collected to characterize the kinetics, local probe concentration does not affect the measurement accuracy. Images in Fig. 5 demonstrate that the phosphorescence lifetime τ (Fig. 5D) is practically independent of the initial intensity I_0 (Fig. 5F). For example, the lifetimes in the region of the smaller polymersome (right and below the main object) are the same as in the larger bright polymersome in the center of the image.

Images of χ^2 and SNR are shown in Fig. 5H and 5I. Interestingly, these two metrics seem particularly sensitive to boundaries; the polymer boundaries seem to appear as a pronounced

ring in both images. It seems that increased scatter and lower phosphorescence at the polymer/solution interface leads to an increased fitting error and thus produces inhomogeneities in χ^2 and SNR images.

The ability to map object morphology by rapid scanning (~50 runs per pixel) can be very useful in practice. For example, when imaging cells or other scattering objects, morphology can be crudely mapped at first, and then accurate point-measurements of oxygen can be taken at selected locations, or smaller subsections of the object can be remapped at longer pixel dwell times. For example, the maximum intensity in the baseline image *b* (Fig. 5G), which includes no phosphorescent signal at all, is 266 counts at 300 runs (compare to 11093 counts in Fig 5A); however it already clearly reveals the object morphology. From these data, we can estimate that ~300 total counts per pixel are sufficient to map the object, which could be obtained using only ~35 runs assuming the same total signal level. The SNR in such data would be insufficient to determine oxygen concentrations, but could provide a useful survey of the object. High resolution oxygen measurements at specific points could be obtained by moving the piezo stage to certain locations of interest.

As mentioned above (see, for example, Fig. 4), demonstration that oxygen concentration inside polymersomes is equivalent to the local oxygen concentration in the medium near polymersomes is of primary importance for the purposes of biological imaging. That is, does oxygen freely diffuse through the polymersome to permit equilibration between the pO_2 inside the vesicle and that in the outer space (e.g. cellular cytoplasm, endosomal compartment)? To answer this question, we imaged a set of polymersomes in the micro-cuvette open to air under ambient conditions (Fig. 6A). Cross-section images of a polymersome attached to the glass surface were performed in different XY planes starting from the bottom of the polymersome (coverslip level) and moving up in 2 μm steps. The data are shown as integrated phosphorescent photons after removing the scatter and the baseline noise. These data also show that the dye is indeed encapsulated within the polymersome and not associated with the membrane surface.

After the scan was completed, glucose oxidase and catalase were added to the cuvette, and the cylinder was covered with a second coverslip. The polymersomes in this case were assembled in solution containing glucose, which served as a substrate for the enzymatic reaction. In this three-component system, glucose oxidase consumes oxygen while converting glucose into gluconic acid, while catalase acts as a scavenger for peroxide produced in the first reaction. The glucose oxidase/catalase effectively deoxygenates glucose-containing aqueous solutions,⁷ as reflected by rapid changes in phosphorescence lifetimes. In our experiment, the enzymes were allowed to act for 15 min, and then Z-scan was repeated from the top down to the coverslip also in 2 μm steps (Fig. 6B). The intensity scale in panels A and B are set to be the same, and the corresponding panels are for the same *z* location.

It is clear from the images that the integrated intensity of the phosphorescence increases dramatically upon deoxygenation of the system, reflecting a decrease in quenching and increase in the phosphorescence lifetime τ . Since the enzymes were added only to the medium outside the polymersomes, and the bulk of the dye is shown to be inside the polymersome, this measurement confirms that the polymersome membranes did not prevent oxygen from diffusing out of polymersomes, and equilibrium was achieved on the time scale of the experiment.

A demonstration that diffusion of oxygen in and out of the vesicles occurs on a biologically relevant time scale comes directly from the kinetics of deoxygenation of the bulk solution as monitored by the phosphorescence of the probe captured inside a polymersome (Fig. 7).

In this experiment, kinetic of the phosphorescence was monitored continuously by imaging the lifetimes in the center ($2 \times 2 \mu\text{m}^2$ region) of a single vesicle. The bulk solution contained glucose and catalase, but no glucose oxidase, and the catalytic process in the beginning was inactive. However, upon addition and dissolution of a small speck of solid glucose oxidase, both the integrated phosphorescence counts (Fig. 7A) and the lifetime (Fig. 7B) rapidly began to increase. The complete removal of oxygen required about ~ 450 s, as evidenced by end of the rise in the phosphorescence signal. The initial lingering of the reaction was probably due to slow dissolution of the enzyme. Taken together, the results in Fig. 4, Fig. 6 and Fig. 7 clearly demonstrate that phosphorescent probes encapsulated inside polymersomes are well suited for probing oxygenation of the biological environments. Provided polymersomes with the probe are delivered inside cells, the probe can act as a robust sensor of intracellular oxygenation.

Three-dimensional imaging of oxygen with high spatial resolution is one of the most attractive features of the two-photon phosphorescence lifetime microscopy. Our earlier demonstration of this capability¹ relied on an experiment with a glass capillary filled with a solution of the phosphorescent probe. The capillary was immersed in an aqueous medium and positioned at an angle relative to the plane of the scanning stage. Unfortunately, due to the strong refraction on the glass/water interface, images of phosphorescence collected at different Z-positions (depths) were poorly-defined and had smeared borders, making 3D rendering difficult. Polymersomes, on the other hand, are well-defined 3D objects, whose membranes are very thin (ca 9.8 nm^{40}) compared to the walls of the glass capillary ($\sim 1 \mu\text{m}^1$). As a result, all the cross-section images in the Z-stack (Fig. 6) were well-defined, allowing for rendering of the first 3D phosphorescence intensity image (Fig. 8).

Estimation of excitation volume

Previous studies of the saturation effect focused on the influence of laser power on the size of the excitation volume for materials with different two-photon absorption cross-sections.⁵³ It was assumed in these studies that the excited state was entirely depopulated between the laser pulses, which is usually the case for fluorescent probes. However, lifetimes of phosphorescent probes are orders of magnitude longer than pulse spacing in high repetition rate Ti:Sapphire oscillators, typically used for excitation in multiphoton imaging. This time mismatch can have a dramatic effect on the excitation volume in two-photon phosphorescence lifetime microscopy. Thus, understanding the photophysics of the system is critical to obtain diffraction limited resolution.

The photophysical processes occurring in PtP-C343 have been analyzed elsewhere.¹ In brief, upon absorption of energy by the two-photon mechanism, the singlet excited state of C343 antenna (A^{S1}) is populated (Scheme 1).

Considering that the rate of stimulated emission ($A^{S1} \rightarrow A^{S0}$) is negligible under the excitation intensities used in our experiments, and the rate of intersystem crossing in PtP ($k_{isc} \sim 10^{12} - 10^{13} \text{ s}^{-1}$)^{61,62} is significantly higher than the rate of FRET ($k_{FRET} \sim 2 \times 10^9 \text{ s}^{-1}$),⁵⁵ Scheme 1 can be reduced to Scheme 2. Since the rate of phosphorescence in PtP-C343 changes by only ~ 3 times throughout the entire range of oxygen concentrations, for simplicity we assume zero-oxygen conditions ($k_p = 1/\tau_0$).

The simulation is divided into two time periods: I) the excitation period, during which excited states A^{S1} and P^{T1} do not significantly depopulate and the only occurring process is two-photon absorption with rate α ; II) the period between the laser pulses, during which fluorescence (k_f), FRET (k_{FRET}) and phosphorescence (k) depopulate states A^{S1} and P^{T1} and re-populate state A^{S0} . Kinetic equations for A^{S0} , A^{S1} and P^{T1} during periods I and II are as follows:¹

$$\text{I: } A^{S_0}(t) = A_0^{S_0} e^{-\alpha t} \quad (4)$$

$$\text{II: } A^{S_1}(t) = A_0^{S_1} e^{-(k_{\text{FRET}} + k_{\text{fl}})t}, \quad (5)$$

$$\text{II: } P^{T_1}(t) = P_0^{T_1} e^{-k_p t} + \frac{A_0^{S_1} k_{\text{FRET}}}{k_{\text{FRET}} + k_{\text{fl}} - k_p} e^{-k_p t} - \frac{A_0^{S_1} k_{\text{FRET}}}{k_{\text{FRET}} + k_{\text{fl}} - k_p} e^{-(k_{\text{FRET}} + k_{\text{fl}})t}, \quad (6)$$

where $A_0^{S_0}, A_0^{S_1}, P_0^{T_1}$ are the concentrations A^{S_0}, A^{S_1} and P^{T_1} at the starts of periods I and II. The remaining solutions, i.e. for A^{S_1} during period I and A^{S_0} during period II, are obtained by considering that at any moment of time $A^{S_0}(t) + A^{S_1}(t) + P^{T_1}(t) = M$, where M is the number of the probe molecules in the volume.

Evolution of A^{S_0}, A^{S_1} and P^{T_1} during the excitation pulse-train can be modeled by applying a sequence of periods I and II, using photophysical rate constants for PtP-C343 and considering that under pure two-photon excitation conditions: $\alpha = \sigma_2 \langle \Phi^2 \rangle$, where Φ is the time averaged square of the photon flux Φ and σ_2 is the two-photon absorption cross-section. The distribution of the time-averaged energy (intensity) in the near-focal volume was modeled using the diffraction theory of Richards and Wolf,⁵¹ which predicts significant deviations from the conventional Airy description⁶³ at high numerical apertures ($\text{NA} > 0.8$). The near-focal volume (typically $6 \times 6 \times 6 \mu\text{m}^3$) in our simulations was divided in an arbitrary number of voxels (typically $\sim 10^8$), and the flux through each voxel was considered constant (see Experimental section and SI for details). In all simulations, excitation trains consisted of 159 pulses, which corresponds to 2 μs -long excitation gates at 80 MHz pulse repetition rate.

The calculated total number of triplet emitters (P^{T_1}) in the volume at the end of the excitation train should differ from the experimentally measured number of phosphorescent photons by several scaling factors, including the phosphorescence quantum yield, collection efficiency, and quantum efficiency of the detector. To facilitate comparisons, calculated and experimental curves (Fig. 9A) we normalized by the values at $p = 0.96 \mu\text{W}$ - a power well within the quadratic range.

The deviation of the calculated power plot from pure quadratic law becomes perceptible at $p \sim 0.75 \mu\text{W}$, similar to that found in the experiment. As the experimental and simulated curves deviate from the quadratic regime, they also deviate from each other. This may arise from higher order non-linear processes that contribute to the experimental signal, but are not included in the simulation, or saturation of the detector at higher powers.

The distribution of the triplet population at the lowest power ($p = 0.96 \mu\text{W}$) matches the distribution of the excitation rate constant α (not shown), which is proportional to the squared distribution of the excitation power. This proportionality is the key feature of the quadratic regime, in which the excitation volume is determined solely by the distribution of the squared excitation flux, resulting in enhanced spatial resolution. A more compact distribution of the excited state population P^{T_1} (blue), as compared to the distribution of the excitation energy (red), is clearly revealed by its both axial (Fig. 9C) and lateral (Fig. 9B) cross-section. This advantage, however, is completely lost in the saturation regime (33.6 μW , black trace), when the distribution of the excited state population expands far beyond the distribution of the excitation energy. Notably, the excitation volume expands especially

strongly in the axial direction (Fig. 9B), which implies that for three-dimensional imaging consideration of saturation is especially important.

In the intermediate cases ($p=14.4\ \mu\text{W}$), corresponding to the experimentally used conditions, there is an increase in the excitation volume compared to the pure quadratic regime, but the increase in the overall signal (e.g. signal at $p=14.4\ \mu\text{W}$ is ~ 180 times stronger than that at $p=0.96\ \mu\text{W}$) can be worth the decrease in resolution. Using full width at half maximum (FWHM) as a metric, the Z-cross section varies from $0.88\ \mu\text{m}$ at $0.96\ \mu\text{W}$ to $1.17\ \mu\text{m}$ at $14.4\ \mu\text{W}$, which is not a very dramatic change in spatial extent for a large signal increase, and still better than the resolution in the one photon excitation regime. In the experiments reported here, polymersomes range from a few microns to tens of microns, so this modest decrease in resolution does not impair the spatial imaging of polymersomes, and in fact, the concomitant increase in signal improves the resolution of the oxygen imaging. It is frequently not appreciated how photophysical parameters beyond the quantum yield of emission and absorption cross-section can have a profound effect on the quality of the image, both in resolution and contrast. Similarly, design of new probe molecules must include careful quantification of the photophysical parameter, both dynamic and static.

The X-Z cross-section ($Y=0$) of the triplet population is shown in Fig. 10 for different excitation powers. It is clear that excitation in the saturation regime leads to a significant expansion of the emitting volume. It should be noted that a recently proposed oxygen imaging method,^{25,64} based on fluorescence correlation spectroscopy (FCS), depends on the saturation effect to modulate the rate of intersystem crossing. FCS methods require explicit knowledge of the excitation volume for data analysis,^{53,64} and in the saturation regime, interpretation of FCS data to determine oxygen concentration is not straightforward. In contrast, in phosphorescence lifetime imaging knowledge of excitation volume is only required for determination of the spatial oxygen resolution, but calculation of oxygen concentration itself depends only on the phosphorescence lifetime and not on the excitation volume.

The saturation effects are important not only in phosphorescence lifetime microscopy, but in all other applications relying on spatially confined generation of triplet states, such as two-photon photodynamic therapy (PDT). Much attention has been given in the recent literature to PDT agents with increased two-photon absorption cross-sections. However, increasing these cross-sections without matching them with appropriate excitation regimes⁶⁵ may jeopardize the main advantage of two-photon excitation, that is the ability to produce the PDT effect in highly localized fashion.

Conclusions

In summary, we have measured the power dependence of the phosphorescence of the probe PtP-C343 under two-photon excitation and established the quadratic excitation power range, which allows for the highest attainable spatial resolution in scanning two-photon phosphorescence lifetime microscopy. We further investigated polymersomes as probe delivery vehicles and determined that neither photophysical nor oxygen quenching properties of the probe are affected upon polymersomal encapsulation. Polymersomes can be loaded with the probe at high concentrations and imaged by two-photon phosphorescence microscopy. Phosphorescence of the probe inside polymersomes responds rapidly to changes in outside oxygenation, making it possible to use probe-loaded polymersomes for measurements in sensitive biological environments, e.g. in cells. A Z-stack of lateral cross-sections of a single polymersome was volume-rendered to give the first microscopic 3D phosphorescence lifetime image. In addition, we showed by way of numerical simulations how the saturation of the probe's triplet state in the vicinity of the laser focus depends on the

excitation regime, and how the latter affects the size of the excitation volume and thus imaging resolution. We illustrated with our own data an example of a case where decreased spatial resolution and increased accuracy of oxygen measurement, is a reasonable compromise. The ability to make these decisions regarding resolution and signal intensity requires detailed understanding of the kinetics of the system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- (1). Finikova OS, Lebedev AY, Aprelev A, Troxler T, Gao F, Garnacho C, Muro S, Hochstrasser RM, Vinogradov SA. *ChemPhysChem*. 2008; 9:1673–1679. [PubMed: 18663708]
- (2). Swartz HM, Clarkson RB. *Physics in Medicine & Biology*. 1998; 43:1957–1975. [PubMed: 9703059]
- (3). Jobsis FF. *Science* (Washington, DC, United States). 1977; 198:1264–7.
- (4). Koch CJ. *Redox Cell Biology and Genetics, Part A*. 2002; 352:3.
- (5). Krishna MC, English S, Yamada K, Yoo J, Murugesan R, Devasahayam N, Cook JA, Golman K, Ardenkjaer-Larsen JH, Subramanian S, Mitchell JB. *Proceedings of the National Academy of Sciences of the United States of America*. 2002; 99:2216. [PubMed: 11854518]
- (6). Ziemer LS, Evans SM, Kachur A, Shuman AL, Cardi CA, Jenkins WT, Karp JS, Alavi A, Dolbier WR, Koch CJ. *European Journal of Nuclear Medicine and Molecular Imaging*. 2003; 30:259. [PubMed: 12552344]
- (7). Vanderkooi JM, Maniara G, Green TJ, Wilson DF. *Journal of Biological Chemistry*. 1987; 262:5476–5482. [PubMed: 3571219]
- (8). Denk W, Strickler JH, Webb WW. *Science* (Washington, DC, United States). 1990; 248:73–6.
- (9). Brinas RP, Troxler T, Hochstrasser RM, Vinogradov SA. *Journal of the American Chemical Society*. 2005; 127:11851–11862. [PubMed: 16104764]
- (10). Finikova OS, Troxler T, Senes A, DeGrado WF, Hochstrasser RM, Vinogradov SA. *Journal of Physical Chemistry A*. 2007; 111:6977–6990.
- (11). Lebedev AY, Troxler T, Vinogradov SA. *Journal of Porphyrins and Phthalocyanines*. 2008; 12:1261–1269. [PubMed: 19763243]
- (12). Discher BM, Won Y-Y, Ege DS, Lee JC-M, Bates FS, Discher DE, Hammer DA. *Science*. 1999; 284:1143–1146. [PubMed: 10325219]
- (13). Golub, A. S, Pittman RN. *American journal of physiology. Heart and circulatory physiology*. 2008; 294:H2905–16. [PubMed: 18375716]
- (14). Wilson DF, Vinogradov SA, Grosul P, Vaccarezza MN, Kuroki A, Bennett J. *Applied Optics*. 2005; 44:5239–5248. [PubMed: 16149347]
- (15). Yaseen MA, Srinivasan VJ, Sakadzic S, Wu W, Ruvinskaya S, Vinogradov SA, Boas DA. *Optics Express*. 2009; 17:22341. [PubMed: 20052157]
- (16). Kindig CA, Kelley KM, Howlett RA, Stary CM, Hogan MC. *Journal of Applied Physiology*. 2003; 94:353. [PubMed: 12391049]
- (17). Sakadzic S, Yuan S, Dilekoz E, Ruvinskaya S, Vinogradov SA, Ayata C, Boas DA. *Applied Optics*. 2009; 48:D169–D177. [PubMed: 19340106]
- (18). Zipfel WR, Williams RM, Webb WW. *Nature Biotechnology*. 2003; 21:1369–1377.

- (19). Diaspro, A.; Sheppard Colin, JR. Confocal and Two-Photon Microscopy: Foundations, Applications, and Advances. Diaspro, A., editor. Wiley-Liss; New York City: 2002.
- (20). Xu C, Webb WW. J. Opt. Soc. Am. B. 1996; 13:481–491.
- (21). Vogel A, Venugopalan V. Chemical Reviews (Washington, DC, United States). 2003; 103:577–643.
- (22). Theer P, Hasan MT, Denk W. Optics Letters. 2003; 28:1022–1024. [PubMed: 12836766]
- (23). Koch CJ. Methods in Enzymology. 2002; 352:3–31. [PubMed: 12125356]
- (24). Koo Y-EL, Cao Y, Kopelman R, Koo SM, Brasuel M, Philbert MA. Analytical Chemistry. 2004; 76:2498–2505. [PubMed: 15117189]
- (25). Opitz N, Rothwell PJ, Oeke B, Schuille P. Sensors and Actuators, B: Chemical. 2003; B96:460–467.
- (26). Xu H, Aylott JW, Kopelman R, Miller TJ, Philbert MA. Analytical Chemistry. 2001; 73:4124–4133. [PubMed: 11569801]
- (27). Springett R, Swartz HM. Antioxidants & Redox Signaling. 2007; 9:1295–1301. [PubMed: 17576162]
- (28). Pawley J. BioTechniques. 2000; 28:2–4.
- (29). Blair S, Katakly R, Parker D. New Journal of Chemistry. 2002; 26:530–535.
- (30). Xu H, Zhang Z. Biosensors & Bioelectronics. 2007; 22:2743–2748. [PubMed: 17360175]
- (31). Im SH, Khalil GE, Callis J, Ahn BH, Gouterman M, Xia Y. Talanta. 2005; 67:492–497. [PubMed: 18970194]
- (32). Borisov SM, Mayr T, Klimant I. Analytical Chemistry (Washington, DC, United States). 2008; 80:573–582.
- (33). Cywinski PJ, Moro AJ, Stanca SE, Biskup C, Mohr GJ. Sensors and Actuators, B: Chemical. 2009; B135:472–477.
- (34). Ma A, Rosenzweig Z. Analytical and Bioanalytical Chemistry. 2005; 382:28–36. [PubMed: 15900448]
- (35). McNamara KP, Rosenzweig N, Rosenzweig Z. Mikrochimica Acta. 1999; 131:57–64.
- (36). Cheng Z, Aspinwall CA. Analyst (Cambridge, United Kingdom). 2006; 131:236–243.
- (37). McNamara KP, Rosenzweig Z. Analytical Chemistry. 1998; 70:4853–4859.
- (38). Brown JQ, McShane MJ. IEEE Sensors Journal. 2005; 5:1197–1205.
- (39). Duchesne TA, Brown JQ, Guice KB, Nayak SR, Lvov YM, McShane MJ. Proceedings of SPIE-The International Society for Optical Engineering. 2002; 4624:66–75.
- (40). Bermudez H, Brannan AK, Hammer DA, Bates FS, Discher DE. Macromolecules (Washington, DC, U. S.). 2002; 35
- (41). Bermudez H, Hammer DA, Discher DE. Langmuir. 2004; 20:540–543. [PubMed: 15773070]
- (42). Ghoroghchian PP, Li G, Levine DH, Davis KP, Bates FS, Hammer DA, Therien MJ. Macromolecules. 2006; 39:1673–1675. [PubMed: 20975926]
- (43). Gomez-Hens A, Manuel Fernandez-Romero J. TrAC, Trends in Analytical Chemistry. 2005; 24:9–19.
- (44). Christian NA, Milone MC, Ranka SS, Li G, Frail PR, Davis KP, Bates FS, Therien MJ, Ghoroghchian PP, June CH, Hammer DA. Bioconjugate Chemistry. 2007; 18:31–40. [PubMed: 17226955]
- (45). Hammer DA, Robbins GP, Haun JB, Lin JJ, Qi W, Smith LA, Peter Ghoroghchian P, Therien MJ, Bates FS. Faraday Discussions. 2008; 139:129–141. [PubMed: 19048993]
- (46). Larson DR, Zipfel WR, Williams RM, Clark SW, Bruchez MP, Wise FW, Webb WW. Science (Washington, DC, United States). 2003; 300:1434–1437.
- (47). Patterson GH, Piston DW. Biophysical Journal. 2000; 78:2159–2162. [PubMed: 10733993]
- (48). Lebedev AY, Filatov MA, Cheprakov AV, Vinogradov SA. Journal of Physical Chemistry A. 2008; 112:7723–7733.
- (49). Abramoff MD, Magelhaes PJ, Ram SJ. Biophotonics International. 2004; 11:36–42.
- (50). Robbins GP, Jimbo M, Swift J, Therien MJ, Hammer DA, Dmochowski IJ. Journal of the American Chemical Society. 2009; 131:3872–3874. [PubMed: 19249827]

- (51). Richards B, Wolf E. Proceedings of the Royal Society of London Series a-Mathematical and Physical Sciences. 1959; 253:358–379.
- (52). Wolf E. Proceedings of the Royal Society of London. Series A, Mathematical and Physical Sciences. 1959; 253:349–357.
- (53). Hess ST, Webb WW. Biophysical Journal. 2002; 83:2300–2317. [PubMed: 12324447]
- (54). Lakowicz, JR. Principles of Fluorescence Spectroscopy. Springer; 2006.
- (55). Finikova OS, Chen P, Ou Z, Kadish KM, Vinogradov SA. Journal of Photochemistry and Photobiology A. 2008; 198:75–84.
- (56). Vinogradov SA, Wilson DF. Applied Spectroscopy. 2000; 54:849–855.
- (57). Lin JJ, Silas JA, Bermudez H, Milam VT, Bates FS, Hammer DA. Langmuir. 2004; 20:5493–5500. [PubMed: 15986691]
- (58). Kostov Y, Harms P, Rao G, Pilato RS. Analyst (Cambridge, United Kingdom). 2000; 125:1175–1178.
- (59). Pfister A, Zhang G, Zareno J, Horwitz AF, Fraser CL. ACS Nano. 2008; 2:1252. [PubMed: 19081748]
- (60). Wu CF, Bull B, Christensen K, McNeill J. Angewandte Chemie-International Edition. 2009; 48:2741.
- (61). Eastwood D, Gouterman M. Journal of Molecular Spectroscopy. 1970; 35:359–75.
- (62). Kim D, Holten D, Gouterman M. Journal of the American Chemical Society. 1984; 106:2793–8.
- (63). Pawley, J. Handbook of Biological Confocal Microscopy. Springer; Singapore: 2006.
- (64). Widengren J, Mets U, Rigler R. Journal of Physical Chemistry. 1995; 99:13368–79.
- (65). Collins H, Khurana M, Moriyama E, Mariampillai A, Dahlstedt E, Balaz M, Kuimova M, Drobizhev M, Yang V, Phillips D, Rebane A, Wilson B, Anderson H. Nature Photonics. 2008; 2:420–424.

Scheme 1.

Photophysical processes occurring in PtP-C343. Light is harvested by the C343 antenna (A) and transferred to the porphyrin core (P) *via* Förster resonance energy transfer. Intersystem crossing produces the triplet state P^{T_1} , which is quenched by oxygen.

Scheme 2.
Simplified kinetic scheme used in simulations.

Figure 1.

Photophysical and quenching properties of PtP-C343 (PBS buffer, pH 7.2, 23.3°C). A. The absorption and emission spectra ($\lambda_{\text{ex}}=460$ nm) of bulk probe dissolved in water at 25 °C. B. Calibration plot showing the dependence of the probe lifetime on partial pressure of oxygen (pO₂).

Figure 2. Power dependencies of phosphorescence of PtP-C343 under ambient conditions: $\log(I_{\text{phos}})$ vs. $\log(p)$. A: in bulk solution (linear fit, slope 1.92), B: inside polymersomes (linear fit, slope 1.92).

Figure 3. Combined phase contrast and wide-field phosphorescence images of polymersomes (not adhered to glass surfaces). Phosphorescence (pink) arises mainly from inside the vesicles. (Image was obtained using Leica TCS SP5 X confocal microscope, 40× oil immersion lens, NA 1.25, pinhole at 1.5 Airy units, emission detection range: 580–740 nm.)

Figure 4. Calibration plots showing the dependence of the probe lifetime on partial pressure of oxygen (pO_2). The oxygen sensing properties of the bulk probe (black) are compared to the oxygen titration plot of the probe inside the polymersome (red). The plots are identical, within the error of the measurements.

Figure 5.

Images of polymersomes in a partially de-oxygenated solution. 40×40 pixel², 8 bit. Imaging parameters: $p=14.4$ μ W, $T_{\text{on}}=3$ μ s, $T_{\text{off}}=497$ μ s, Number of runs: 300. Scale bar (panel I): to 5 μ m. Images as defined in Experimental section: A) I_{tot} ; B) I_{phos} ; C) I_{calc} ; D) τ ; E) $p\text{O}_2$; F) I_0 ; G) b ; H) χ^2 ; I) SNR.

Figure 6.

Z-scan of a polymersome attached to the glass surface, $p=14.4 \mu\text{W}$. Image shows phosphorescent photons after removal of the scatter and the background. XY cross-sections are taken in $2 \mu\text{m}$ steps in Z from the bottom up (left-to-right, top-to-bottom in the images). A) Polymersomes in a cuvette equilibrated with air. B) The same sample imaged after addition of glucose oxidase/catalase enzymatic system to deoxygenate the solution. The image marked with a star is the same image analyzed in depth in Fig. 7. Scale bar - $5 \mu\text{m}$.

Figure 7. Oxygen removal from bulk solution by enzymatic system (glucose/glucose oxidase/catalase) as monitored by phosphorescence from a single polymersome. $p=9.6 \mu\text{W}$ A) Integrated intensity of phosphorescence ($I_0\tau$). B) Phosphorescence lifetime.

Figure 8. Three-dimensional phosphorescence intensity image of a single polymersome vesicle obtained by rendering cross-section images from Fig. 8B. $p=14.4 \mu\text{W}$.

Figure 9.

Influence of saturation on power dependence and resolution. A. Normalized calculated (red) and experimental (blue) power dependencies of phosphorescence of PtP-C343 for the same excitation conditions (see Experimental section for detail). X- (B) and Z- (C) cross-sections of excitation energy distribution and triplet populations (P^{T1}) created by high repetition-rate pulse trains at different average powers p . Experimental imaging was performed $p=14.4 \mu\text{W}$ (Fig. 7, 8 & 10).

Figure 10.

Normalized images of the X-Z cross-section ($Y=0$) of the near-focal volume at different excitation powers. Scale bar is $1\ \mu\text{m}$. A. Distribution of excitation energy. B. Distribution of excited triplet state for $p=0.96\ \mu\text{W}$ (pure quadratic regime). C. Distribution of excited triplet state for $p=14.4\ \mu\text{W}$. (This power was typically used in imaging experiments described in this paper.) D. Distribution of excited triplet state for $p=33.6\ \mu\text{W}$ (well outside the quadratic regime).