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Design of Metalloporphyrin-Based Dendritic Nanoprobes for Two-Photon Microscopy of Oxygen

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Abstract

Metalloporphyrin-based phosphorescent nanoprobes are being developed for two-photon microscopy of oxygen. In these molecular constructs generation of porphyrin triplet states upon two-photon excitation is induced upon the intramolecular Förster-type resonance energy transfer from a covalently attached 2P antenna. In the earlier developed prototypes, electron transfer between the antenna and the metalloporphyrin strongly interfered with the phosphorescence, reducing the sensitivity and the dynamic range of the sensors. By tuning the distances between the antenna and the core and adjusting their redox potentials the unwanted electron transfer could be prevented. An array of phosphorescent Pt porphyrins (energy transfer acceptors) and 2P dyes (energy transfer donors) was screened using dynamic quenching of phosphorescence, and the FRET-pair with the minimal ET rate was identified. This pair, consisting of Coumarin-343 and Pt *meso*-tetra-(4-alkoxyphenyl)porphyrin, was used to construct a probe in which the antenna fragments were linked to the termini of G3 poly(arylglycine) (AG) dendrimer with PtP core. The folded dendrimer formed an insulating layer between the porphyrin and the antenna, simultaneously controlling the rate of oxygen quenching (Stern-Volmer oxygen quenching constant). Modification of the dendrimer periphery with oligoethyleneglycol residues made the probe's signal insensitive to the presence of proteins and other macromolecular solutes.

Keywords

porphyrin; phosphorescence; oxygen; two-photon; FRET; photoinduced electron transfer; triplet state

INTRODUCTION

Non-invasive measurement and imaging of oxygen distributions (pO_2) is important for many areas of biological research. Nevertheless, methods for oxygen imaging in biological systems are not yet adequately developed. Oxygen-dependent quenching of phosphorescence [1] is a minimally invasive optical technique, offering superior sensitivity, selectivity and excellent temporal resolution. Using phosphorescent probes with controllable quenching parameters and defined bio-distributions [2], phosphorescence quenching has been implemented as 2D [3] and 3D tomographic [4] imaging. A new high-resolution variant of the method, based on the combination of phosphorescence quenching and two-photon laser scanning microscopy (2P LSM) [5], is currently being developed [6]. In this article, we review the principles of phosphorescent probes for two-photon microscopy of oxygen and discuss the synthesis and

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photophysics of a newly developed probe, which enabled the first high-resolution oxygen imaging experiments by 2P LSM.

RESULTS AND DISCUSSION

General principles

The rationale for construction of two-photon enhanced phosphorescent nanoprobe has been discussed in our previous publications [7,8]. In brief, our design makes use of the intramolecular Förster-type resonance energy transfer (FRET) from several two-photon (2P) dyes (2P antenna) onto Pt or Pd porphyrins (phosphorescent cores) with the purpose to increase their apparent 2P action cross-sections [9,10]. In the originally developed prototypes, photoinduced electron transfer (ET) between the core metalloporphyrin and 2P-antenna chromophores significantly interfered with the phosphorescence, diminishing the probe performance [7,8]. In addition, some phosphorescent metalloporphyrins could undergo linear excitation directly into their triplet states (T_1), especially when the corresponding $S_0 \rightarrow T_1$ bands occurred in the proximity of the Ti:Sapphire laser spectrum [8]. Taking these results into account, we identified a set of requirements for optimized 2P-enhanced phosphorescent nanosensors:

1. the phosphorescent chromophore should possess high emission quantum yield and appropriate triplet lifetime to allow measurements in the physiological range of oxygen concentrations (0–160 mm Hg or $0\text{--}2.0 \times 10^{-4}$ M in aqueous solutions at 37 °C);
2. the phosphorescent chromophore should not be excitable *via* single-photon (1P) mechanism in the wavelength range used for the excitation of 2P antenna;
3. the 2P-antenna chromophores should possess large two-photon absorption (2PA) cross-sections (σ_2), preferably within the tunability range of commercial Ti:Sapphire lasers; the emission of the antenna must overlap with absorption bands of the phosphorescent cores;
4. 2P-antenna/core FRET pair should be not active in photoinduced ET reactions;
5. 2P-antenna chromophores should be as small as possible, so that when several of them are attached to the core the entire construct retains the same general architecture and possesses good solubility.

Phosphorescent chromophore—Pt *meso*-tetraarylporphyrins (PtP's) were chosen as phosphorescent cores due to their strong phosphorescence at ambient temperatures and intense visible absorption bands (Q-bands), overlapping with fluorescence of many 2PA dyes [11]. Compared to more commonly used Pd porphyrins [1–4], PtP's exhibit higher phosphorescence quantum yields ($\phi_p \sim 0.10$) and shorter triplet lifetimes ($\tau_0 \sim 40\text{--}50$ μ s at 23 °C). Although longer phosphorescence lifetimes τ_0 increase oxygen sensitivity (*vide infra*), measurements of long lifetimes necessitate longer acquisition periods. For scanning microscopy applications this might present a problem. On the other hand, very short lifetimes, such as those of Ru (bpy) $^{2+}$ and similar complexes ($\tau_0 \sim 1$ μ s) [12], are inadequate for accurate measurements in the physiological pO_2 range.

Previous studies of Pt complexes of tetrabenzoporphyrins [8], which possess higher intrinsic 2PA cross-sections than regular porphyrins, revealed that these tetrapyrroles exhibit unusually strong $S_0 \rightarrow T_1$ absorption bands ($\epsilon \sim 100$ M $^{-1}$.cm $^{-1}$). Regular (non-extended) PtP's also possess $S_0 \rightarrow T_1$ bands [13], but these are significantly blue-shifted relative to the 2PA maxima of dyes capable of FRET onto PtP's.

2P antenna dyes and dynamic quenching experiments—From a large number of known 2P dyes we selected a few candidates with emission spectra overlapping with absorption bands of PtP's in order to allow efficient FRET. A set of such dyes and PtP's with variable redox potentials were examined using dynamic quenching of phosphorescence [11]. These experiments were carried out with the purpose to identify a dye pair with as low as possible propensity to the triplet quenching by photoinduced ET. Coumarin-343 (C343) was chosen because of its highest oxidation potential, relatively large 2P action cross-section ($\eta = \sigma_2 \phi_{fl}$) at 840 nm ($\sigma_2 = 28 \text{ GM}$, $\phi_{fl} = 0.90$) [14], small molecular size (MW 285) and ease of derivatization [7]. Simultaneously, we found that PtP modified with 4-alkoxyphenyl groups was the least efficient electron acceptor in the photoinduced ET reaction involving C343 as a reducing agent.

Dendritic encapsulation—Quenching of metalloporphyrin phosphorescence by oxygen is a diffusion-controlled process. The rate of oxygen diffusion to the triplet state chromophore is represented by the value of quenching constant k_q in the Stern-Volmer equation (Eq. 1).

$$1/\tau = 1/\tau_0 + k_q \times pO_2, \quad (1)$$

where τ is the phosphorescence lifetime at oxygen pressure pO_2 . The k_q 's for unprotected PtP's in aqueous solutions are typically too high, 1,500–2,000 $\text{mm Hg}^{-1} \cdot \text{s}^{-1}$, making lifetime measurements at ambient pO_2 's impossible due to the excessively strong quenching. Encapsulation of metalloporphyrins into dendrimers makes it possible to attenuate the quenching constants, so that at ambient oxygen pressures probes exhibit adequate emissivity [2,15,16]. Such attenuation is most effectively achieved when dendritic wedges are anchored at the *meta*-positions of the *meso*-aryl rings of tetraarylporphyrins [16]. In the case of *para*-attachment, much larger dendritic wedges are required for complete encapsulation [17].

By far the most critical parameter with respect to the encapsulation efficiency is the composition of the dendritic matrix [16]. Dendrimers composed of aromatic building blocks, e.g. Fréchet-type dendrimers [18], are able to reduce k_q 's by 10–100 times already at early dendritic generations (G1–G2), while polyglutamic dendrimers [15] or Newkome-type poly (ester amide) dendrimers [19] of comparable sizes are much less effective in restraining oxygen diffusion. Recently developed poly(aryl-glycine) (AG) dendrimers [20] appear to be superior to the other dendrimers with aromatic skeletons in terms of practical construction of phosphorescent oxygen probes [21].

Attempts to use Pt *meso*-tetra(3,5-dicarboxyphenyl)porphyrin [7] as a model core in combination with Generation 2 and 3 (G2, G3) AG-dendrons and several peripheral 2P-antenna units (C343) yielded probes almost entirely lacking oxygen sensitivity. Oxygen quenching constants of these probes were found to be extremely low, 30–50 $\text{mm Hg}^{-1} \cdot \text{s}^{-1}$ (at 23 °C). The combination of the relatively short intrinsic lifetime of PtP ($\tau_0 = 45 \mu\text{s}$), strong shielding effect of folded AG-dendrons and extra protection by C343 units led to a very narrow dynamic range of lifetimes, i.e. only several μs 's. Subsequently, we chose in favor of PtP's with *para*-positioned dendritic substituents, which allowed easier access of oxygen to the cores.

To evaluate the effect of dendritic branches as “insulators” from ET, Pt *meso*-tetra(4--alkoxyphenyl)porphyrin-based AG dendrimers of three successive generations (G1–G3) were synthesized and decorated at the periphery with several C343-antenna fragments. G3 branches were determined to have the optimal size, as they allowed adequate separation between C343 units and the core, provided necessary protection from oxygen and made it possible to use more solubilizing methoxyoligoethyleneglycol (PEG) groups for modification of the dendrimer

periphery. We also found that using PEG350 units (monomethoxypolyethyleneglycol, Av MW 350) did not result in the desired high aqueous solubility, whereas using longer PEG fragments, such PEG750 (Av. MW 750), gave probes with no evidence of aggregation in aqueous solutions.

Synthesis

The overall synthesis of 2P-enhanced phosphorescent probe PtP-C343 is shown in Scheme 1. The detailed description of the procedures can be found in ref. [6]. The following abbreviations for dendritic molecules are used throughout the text:

For dendrons: **X-AGⁿR**, where AG denotes the dendritic aryl-glycine skeleton, *n* is the dendrimer generation number, X is the focal functionality and R is the terminal group. For example, butyl-ester terminated AG-dendron of generation 2 with Boc-protected amino group at the focal point is abbreviated BocNH-AG²OBu.

For dendrimers: **C-(AGⁿR)_m**, where C denotes the dendrimer core, AG is the dendritic aryl-glycine skeleton, *n* is the generation number, R is the terminal group and *m* is the number of dendritic wedges attached to the core. For example, generation 2 AG-dendrimer consisting of Pt porphyrin core (PtP) and four dendrons terminated by carboxyl groups is abbreviated as PtP-(AG²OH)₄.

Synthesis of PtP (**3**) was described earlier [11]. Upon hydrolysis **3** gave tetracarboxylic acid **4**, which reacted with four H₂N-AG³OBu dendrons [**5**] in a 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/diisopropylethylamine (DIPEA)-catalyzed reaction, yielding dendrimer PtP-(AG³OBu)₄ (**6**) in 90% yield. Polycarboxylic acid **7** was obtained in 94% yield upon the gentle base-mediated hydrolysis of **6**. Compound **7** and all its precursors were unambiguously characterized by standard analytical methods. MALDI spectra of the dendrimers revealed monodisperse molecular distributions.

Modification of the periphery of **6** with ethylenediamine derivatives of C343 (**8**) [7] was carried out in the divergent fashion, using HBTU/DIPEA-catalyzed peptide coupling reaction. Based on the MALDI-TOF and UV-vis spectra, dendrimer **9** contained on average 4.7 C343 moieties per PtP. Esterification of **9** by PEG750 groups was accomplished by an earlier published method [22], using N,N'-dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBT) as a catalyst. Probe PtP-C343 was isolated as a viscous orange-brown solid with high aqueous solubility.

Photophysical characterization

The photophysical data for probe PtP-C343 are summarized in Table 1. All the measurements were performed using instrumentation described in our previous publications [7,8].

Compounds **4** and **8** (Scheme 1) were used as references to estimate the rate and the efficiency of the FRET. The absorption and emission spectra of these chromophores are shown in Fig. 1.

To facilitate the comparison with the literature data, the phosphorescence quantum yield (ϕ_p) of **4** is expressed in Table 1 relative to the fluorescence of *meso*-tetraphenylporphyrin (H₂TPP), measured in deoxygenated benzene ($\phi_f = 0.11$) [23]. On the contrary, the fluorescence quantum yield of **8** (ϕ_f) is expressed relative to the fluorescence of Rhodamine B in MeOH ($\phi_f = 0.50$) [24]. It should be mentioned that when compared to the fluorescence of H₂TPP, compound **8** exhibits the quantum yield significantly higher than 1.0, indicating that the value 0.11 for ϕ_f of H₂TPP is exaggerated.

Energy and electron transfer processes in the probe molecule—The Förster distance r_0 for chromophores **8** (donor) and **4** (acceptor) in water (refractive index $n_d = 1.33$) is ca. 4.6 nm, based on the experimentally measured fluorescence quantum yield of **4** ($\phi_f = 0.95$), spectral overlap integral ($J = 5.72 \times 10^{-14}$) and assuming random orientation of the transition dipoles ($\kappa^2 = 2/3$):

$$r_0 = \sqrt[6]{\frac{9000 \ln(10) \kappa^2 \phi_f J}{128 \pi^2 N_a n_d^4}} \approx 4.6 \times 10^{-7} \text{ cm} \quad (2)$$

In Eq. 2, N_a denotes the Avogadro number.

Molecular dynamics simulations (CHARMM force field) were performed using compound **9** as a model (Fig 2).

Simulations were performed in the medium with distance-dependent dielectric (scale factor = 4). In the conformations sampled, distances between C343 and PtP varied from $\sim 40 \text{ \AA}$ (fully extended branches) to as low as $\sim 5 \text{ \AA}$. The average distance (sampled over 10 conformations) was found to be $r_{\text{FRET}} \sim 20 \text{ \AA}$, at which the rate constant for the FRET was calculated as:

$$k_{\text{FRET}} = k_f \left(\frac{r_0}{r_{\text{FRET}}} \right)^6 \approx 3.98 \times 10^{10} \text{ s}^{-1}, \quad (3)$$

Here, $k_f = 1/\tau_f = 2.5 \times 10^8 \text{ s}^{-1}$, where $\tau_f = 4.0 \times 10^{-9} \text{ s}$ is the experimentally determined fluorescence lifetime of **8** in aqueous solution at pH 7.2. At this rate FRET should occur with >0.99 efficiency.

The experimental efficiency of the FRET (ϕ_{FRET}) was determined by comparing its absorption and excitation spectra (Fig. 1C), recorded for the lowest energy emission maximum, i.e. phosphorescence of PtP ($\lambda_{\text{max}} = 682 \text{ nm}$). From these measurements, the FRET quantum yield was found to be $\phi_{\text{FRET}} = 0.68$, which is significantly lower than predicted by the calculations. Time resolved measurements revealed that only a very small fraction of C343 fluorescence in the steady-state emission spectra of PtP-C343 (Fig. 1B) originated from an impurity. The main component of the decay of C343 fluorescence in PtP-C343 had the lifetime $\tau_f = 210 \text{ ps}$. In the absence of other quenching processes, this value would correspond to the FRET rate of about $3.1 \times 10^9 \text{ s}^{-1}$, which is more than ten times lower than predicted by the Förster model (Eq. 3). Such low value could be explained by incomplete orientational averaging due to the restricted mobility of the chromophores attached to a folded dendrimer; but still, even at this low rate the quantum yield of FRET would be 0.92, but not 0.68, as suggested by the steady-state measurements (Fig. 1C). Therefore, it appears that another quenching process operates in the molecule of PtP-C343. This process is likely to be the ET from C343 first excited singlet state S_1 onto PtP. To account for the experimental value of $\phi_{\text{FRET}} = 0.68$, the rate of this ET would have to be $k_{\text{ET}} \sim 8.0 \times 10^8 \text{ s}^{-1}$.

An obvious question arises as to why the ET between C343 and PtP so effectively competes with such a fast process as FRET while having only a minor effect on the emission from the long-lived triplet state? Indeed, the phosphorescence quantum yield of PtP-C343 was found to be 0.10, which is only slightly below that of reference compound **4** ($\phi_p = 0.13$). This fact implies that the rate of ET involving the PtP triplet state T_1 and the ground state S_0 of C343 is comparable to that of the phosphorescence, i.e. $\sim 10^3\text{--}10^4 \text{ s}^{-1}$.

The answer most likely has to do with the difference between the driving forces of the two ET processes involving different intermediates (Fig. 3).

The driving force for ET1, which involves S_1 -state of C343 and the ground state of PtP is ca. 0.8–0.9 eV larger than the driving force of ET2 between T_1 -state of PtP and the ground state of C343. In fact, ET2 may be even slightly endergonic. Such difference in ΔG 's arises from the large Stokes shift of the C343 fluorescence (0.26 eV), explained by significant stabilization of its singlet state by the solvent, as well as from a much larger singlet-triplet splitting ($2J$) within the PtP molecule (0.6 eV) than within the charge separated complex $[\text{PtP}^--\text{C343}^+]$ [25,26,27].

Phosphorescence—The phosphorescence decay recorded from an aqueous solution of PtP-C343 (pH 7.2) and its underlying phosphorescence lifetime distribution, recovered by a Maximum Entropy algorithm [28], are shown in Fig. 4. The main peak of the distribution is centered at $\tau \sim 60 \mu\text{s}$, but substantial fraction of the probe decays at a much higher rate, i.e. $\tau \sim 14 \mu\text{s}$. Existence of this fast decay is consistent with a sub-population of molecules in which some C343 units are positioned close to PtP. In this population, ET2 (Fig. 3) operates at its maximal rate, reducing the overall yield of the phosphorescence emission.

The short-lived component in the phosphorescence lifetime spectrum causes the corresponding Stern-Volmer plots to be somewhat non-linear. Nevertheless, the curvatures of the plots were found to be constant, giving highly reproducible calibration curves. Phosphorescence quenching plots for PtP-C343 in the presence and in the absence of albumin (A) at different pH (B) and different temperatures (D) are shown in Fig. 5.

It is clear from the graphs that neither changes in pH nor the presence of large proteins with lipophilic binding sites, such as albumin, affect the probe calibrations. Such behavior is the key to the probe's specificity to oxygen. It is worth mentioning that insensitivity of the probe's constants to the presence of endogenous macromolecular objects (proteins, cellular membranes etc) is not easily achievable. The majority of known probes interact with macromolecules by way of non-specific binding, causing large changes in the accessibility of the chromophores to oxygen and alterations in oxygen quenching constants. As a result, oxygen measurements with unprotected probes cannot be interpreted quantitatively.

The apparent oxygen quenching constants k_q , derived from the plots shown in Fig. 5, are in the range of $130\text{--}160 \text{ mm Hg}^{-1}.\text{s}^{-1}$, depending on the temperature. Such values are suitable for oxygen measurements in the physiological $p\text{O}_2$ range, although higher constants (i.e. $300\text{--}400 \text{ mm Hg}^{-1}.\text{s}^{-1}$) would be preferable. The combined effect of the dendrimer, peripheral C343-units and PEG residues in PtP-C343 brings about ~ 15 -fold attenuation in the rate of the oxygen diffusion. Using smaller and/or more oxygen-permeable dendritic shells, e.g. polyglutamic [15] or poly(ester-amide) Newkome-type dendrimers [19], in combination with chromophores with longer intrinsic decay times, e.g. Pd-porphyrins ($\tau_0 = 300\text{--}700 \mu\text{s}$), is likely to result in higher oxygen sensitivity. The trade-off, however, is lower quantum yields of Pd porphyrins, and, as already mentioned, longer acquisition periods.

Two-photon excitation—The two-photon excitation experiments were designed to evaluate the degree of the phosphorescence enhancement *via* FRET from the 2P-antenna. Using the method of relative fluorescence and Rhodamine B as a standard [14,24], we determined that the 2PA cross-section of C343 (**8**) in aqueous buffered solution (pH 5.5) has the value of 28 GM, which is consistent with earlier reports. The σ_2 value for core PtP (**4**) was found to be close to the previously measured values ($\sigma_2 \sim 5 \text{ GM}$) of other Pt porphyrins [7] and similar tetrapyrroles [29,30].

Measuring σ_2 of PtP-C343 by registering explicitly the phosphorescence signal upon 2P excitation is complicated due to the saturation effects, caused by the mismatch between the lifetime the triplet state and a much shorter interval between the pulses of high repetition rate Ti:Sapphire lasers (e.g. 10 ns at 100 MHz) [31], typically used in imaging. On the other hand, measurements at low excitation powers, i.e. below the saturation onset, are subject to large errors due to low signal-to-noise ratios (SNR). Using C343-fluorescence signal to estimate the 2PA cross-section of PtP-C343 also suffers from low SNR's, since C343 fluorescence in PtP-C343 has low quantum yield (Table 1).

The plots in Fig. 6 illustrate how the probe's signals change with averaged laser power under excitation by high repetition rate pulses from a Ti:Sapphire oscillator (80 MHz rep. rate, $\lambda_{ex} = 840$ nm, 110 fs). The fluorescence signal ($\tau_f = 210$ ps) exhibits nearly perfect quadratic power dependence (Fig. 6A), whereas the phosphorescence ($\tau_p = 60$ μ s) visibly deviates from the quadratic plot already at quite low incident powers (Fig. 6B). Nevertheless, in the region where the quadratic behavior is still apparent ($< \sim 15$ mW), the phosphorescence of PtP-C343 was found to be enhanced about 23-fold compared to the reference Pt porphyrin (**4**) without the 2P-antenna. This enhancement is the key to successful oxygen imaging.

From the point of view of imaging resolution, it is very important to carry out measurements in the regime where the dependence of the signal on the excitation power is non-linear. Non-linear dependence of the concentration of the emitting species in the laser focal volume on the excitation flux is the key to the high spatial resolution of 2P LSM [5]. Assuming pure 2P mechanism, the rate constant of the absorption (α) is proportional to the time-averaged square of the excitation flux ($\langle \Phi^2 \rangle$) with the proportionality coefficient σ_2 . The emission rate constant (k_{emiss}) on the contrary is determined only by the nature of the excited state, e.g. for the triplet state of PtP it is quite low (10^4 – 10^5 s $^{-1}$). At such a low rate of depletion, PtP-C343 in the focal volume simply does not have enough time to relax back to the ground state between rapidly arriving excitation pulses. Only if the flux Φ and/or the 2PA cross-section σ_2 are very low, the rate of the triplet generation becomes comparable to that of the emission ($\alpha \approx k_{emiss}$). Once this limit is exceeded, the fraction of the triplet state molecules in the excitation volume rapidly reaches saturation, and further increase in the excitation power leads only to the increase of the excitation volume and loss of resolution.

These effects should be taken into account not only when designing triplet imaging probes but in other applications where spatially confined generation of triplet states is important, such as 2P PDT [32]. Increasing 2PA cross-sections of 2P chromophores without matching them with appropriate excitation regimes can jeopardize the main advantage of the 2P excitation, that is high spatial localization of the excitation volume.

CONCLUSIONS

We have demonstrated that a phosphorescent nanoprobe for 2P oxygen microscopy can be constructed using a dendritically encapsulated Pt porphyrin, decorated at the periphery with 2P-antenna chromophores. The latter enhance the 2PA-induced phosphorescence of the porphyrin *via* intramolecular FRET. The new probe is currently being used in cellular 2P oxygen imaging experiments.

Acknowledgments

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Fig 1.

A - Absorption and emission spectra of reference chromophores **4** and **8** in deoxygenated NMP solution. Fluorescence of **8** ($\phi_f = 0.95$) and phosphorescence of **4** ($\phi_p = 0.13$) are not scaled. **B** - absorption and emission spectra ($\lambda_{excit} = 460$ nm) of PtP-C343 in deoxygenated NMP. **B** - absorption and excitation spectra ($\lambda_{emiss} = 700$ nm) of PtP-C343 in deoxygenated aqueous solution.

Fig 2.
Compound **9** used as a model for determining average FRET distances (r_{FRET}) between C343 antenna chromophores and PtP core in PtP-C343.

Fig 3.

Qualitative state diagram of energy and electron transfer processes occurring in the molecule of PtP-C343. *isc* - intersystem crossing in PtP.

Fig 4.
A - decay of phosphorescence of PtP-C343; **B** - underlying phosphorescence lifetime distribution obtained by the Maximum Entropy Method.

Fig 5.

Stern-Volmer oxygen quenching plots of probe PtP-C343 in phosphate buffered solutions (10 mM). **A** - in the absence and presence (2%) of bovine serum albumin; **B** - at different pH; **C** - at different temperatures.

Fig 6.

Emission power dependencies of PtP-C343 and reference chromophores: **A** - fluorescence of C343 units in PtP-C343 and its fit to a quadratic function; **B** - phosphorescence of PtP in PtP-C343 and its fit to a function; **C** - phosphorescence of PtP in PtP-C343 and the reference PtP (4).

Scheme 1.

Synthesis of 2P-enhanced phosphorescent probe PtP-C343.

Table 1

Photophysical data for PtP-C343

Parameter	Value
$\lambda_{\max}$ abs, nm, (ϵ ($M^{-1}.cm^{-1}$))	407 (290,000) 450 (166,306) 515 (26,500)
$\lambda_{\max}$ emiss, nm	498 (f); 682 (p)
ϕ_f (C343 fluorescence)	0.011^a (0.004) ^b
ϕ_p (PtP phosphorescence) ^c	0.10^a (0.042) ^b
τ_f (ns)	0.21
τ_0 (μs) ^c	60
ϕ_{FRET}	0.68
σ_2 (GM) ^d (1 GM = $10^{-50} cm^4.s.photon^{-1}$)	130
k_q (mm Hg ⁻¹ .s ⁻¹) ^e	170

^a measured in N-methylpyrrolidone (NMP) relative to fluorescence of H₂TPP in deox. C₆H₆, $\phi_f = 0.11$ [23].

^b measured in (NMP) relative to fluorescence of Rhodamine B in MeOH, $\phi_f = 0.50$ [24].

^c measured in aqueous solution, deoxygenated by glucose/glucose oxidase/catalase enzymatic system [1].

^d calculated as a sum of 2PA cross-sections of C343-units ($\sigma_2 = 28$ GM) [14] and PtP ($\sigma_2 = 4$ GM) [7].

^e measured at 37 °C.