Video Article

Synthesis and Calibration of Phosphorescent Nanoprobes for Oxygen Imaging in Biological Systems

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Abstract

Oxygen measurement by phosphorescence quenching [1, 2] consists of the following steps: 1) the probe is delivered into the medium of interest (e.g. blood or interstitial fluid); 2) the object is illuminated with light of appropriate wavelength in order to excite the probe into its triplet state; 3) the emitted phosphorescence is collected, and its time course is analyzed to yield the phosphorescence lifetime, which is converted into the oxygen concentration (or partial pressure, pO_2). The probe must not interact with the biological environment and in some cases to be 4) excreted from the medium upon the measurement completion. Each of these steps imposes requirements on the molecular design of the phosphorescent probes, which constitute the only invasive component of the measurement protocol. Here we review the design of dendritic phosphorescent nanosensors for oxygen measurements in biological systems. The probes consist of Pt or Pd porphyrin-based polyarylglycine (AG) dendrimers, modified peripherally with polyethylene glycol (PEG's) residues. For effective two-photon excitation, termini of the dendrimers may be modified with two-photon antenna chromophores, which capture the excitation energy and channel it to the triplet cores of the probes via intramolecular FRET (Förster Resonance Energy Transfer). We describe the key photophysical properties of the probes and present detailed calibration protocols.

Video Link

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Protocol

1. General description of oxygen measurement protocol

(This section does not have any action, but is crucial for understanding the rest of the paper. It can be filmed, for example, as a sequence of a few Power Point slides, accompanied by the voice.)

1.1) The probe is delivered into the medium of interest, for example, injected in the blood or interstitial fluid of an animal.

1.2) The object (surface of the tissue) is illuminated with the light of appropriate wavelength in order to promote the probe into its excited triplet state. Typically excitation occurs *via* the one-photon mechanism. However, in the case of special two-photon-enhanced probes, excitation can be done *via* the two-photon mechanism, which permits using the probes in high-resolution microscopy applications. One-photon excitation generally provides less spatial resolution, but requires simpler instrumentation and can be used in single-point measurements with LED-based fiber-optic phosphorometers.

1.3) Phosphorescence emitted by the probe originates from the long-lived triplet state. The probe molecule must be specially designed to give high quantum yield of the triplet state and emit phosphorescence instead of fluorescence. While in the triplet state, the probe can experience collisional encounters with molecules of oxygen, which can de-activate the triplet state - a process called "quenching." In the absence of oxygen, the lifetime of the triplet state, and hence of the phosphorescence decay, is τ_0. In the presence of oxygen, the phosphorescence lifetime (τ) is shortened as a result of the quenching. There is a direct relationship between the lifetime of the triplet state and the amount of oxygen in the environment. This is the well-known Stern-Volmer relationship, which connects the lifetime (τ) of the phosphorescence to oxygen concentration [O_2] (or oxygen partial pressure pO_2):

$$\frac{\tau_0}{\tau} = 1 + k_q \tau_0 [O_2]$$

In biological systems, oxygen is by far the most effective quencher of phosphorescence, which makes the phosphorescence quenching method very selective to oxygen.

1.4) In order to measure the lifetime t, the emitted phosphorescent photons are binned in time. For example, after the excitation pulse, 200 photons might be collected in the first 15 microseconds, 150 photons collected in the next 15 microseconds, and so on, until no photons are collected. The numbers in the bins plotted against time give the phosphorescence decay, which is analyzed to yield the lifetime τ. In imaging, this procedure is applied in every pixel of the image, resulting in phosphorescence lifetime maps. Measurements of lifetimes are insensitive to the heterogeneities of probe distribution throughout the object, which is common for biological samples. Thus, lifetimes report only on oxygen. Lifetimes τ are converted into oxygen concentrations using the Stern-Volmer relationship.

2. Construction of phosphorescent probes

Phosphorescent probes for oxygen measurements in biological systems consist of phosphorescent chromophores (cores), encapsulated into cages constructed of dendrimers - highly regular hyperbranched polymers [3]. Dendrimers protect the cores from interactions with the environment and control the rate of oxygen diffusion to the cores, making it possible to optimize the sensitivity and dynamic range of the probes (constant k_q in the Stern-Volmer relationship). The termini of the dendrimers are modified with chemically inert hydrophilic polyethylene glycol (PEG) groups, making the probes highly water-soluble and preventing their interactions with biological macromolecules.

Phosphorescent cores of the probes are Pt or Pd complexes of porphyrins and π-extended porphyrins. π-Extended porphyrins are porphyrins in which pyrrole rings are fused with external aromatic fragments. π-Extension allows tuning of the probes spectroscopic properties to satisfy the requirements of a particular imaging application (e.g. microscopy vs. tomography). As an example, we describe synthesis of Pd tetaaryltetrabenzoporphyrins ($PdAr_4TBP$) [4]. $PdAr_4TBP$'s have powerful absorption bands in the far-red part of the spectrum and strong phosphorescence, well suited for biological oxygen measurements.

The synthesis of $PdAr_4TBP$'s consists of the following steps:

2.1) Preparation of tetraaryltetracyclohexenoporphyrins (Ar4TCHP) by Lindsey condensation [5] of tetrahydroisoindole with substituted benzaldehydes. To a 0.01 M solution of tetrahydroisoindole (1 eq) in CH_2CL_2 the aromatic aldehyde (1 eq) was added under argon. The reaction mixture was stirred for 10 min in the dark at R.T. BF_3xEt_2O (0.2 eq) was added, and the reaction mixture was stirred at rt for an additional 2 h. DDQ (1 eq) was added, and the mixture was left overnight under continuous stirring. The solution was washed with 10% aq Na_2SO_3, 10% aq Na_2CO_3, 5% aq HCl and finally with brine. The organic phase was dried over Na_2SO_4, then concentrated in vacuum. The residue was recrystallized from CH_2Cl_2 - *tert*-butyl ether mixture to give Ar_4TCHP as green powder. Yields are about 50%.

2.2) Insertion of Pd to obtain $PdAr_4TCHPs$. Free-base porphyrins (1 eq) were treated with $PdCl_2$ or $Pd(OAc)_2$ (1.2 eq) in refluxing CH_3CN in the presence of excess of Et_3N (20 eq). The conversion was monitored by UV-vis spectroscopy (solvent CH_2Cl_2-AcOH) and considered complete after the Soret band of dictation at 468-472 nm disappeared. The mixture was allowed to cool down, diluted with CH_2Cl_2 and filtered through a thin layer of Celite to remove Pd(0). The solvent was evaporated, and the residue was purified by chromatography on silica gel column using CH_2Cl_2 as a solvent. Dark red fraction was collected, the solvent was evaporated to give target $PdAr_4TCHPs$ in almost quantitative yield.

2.3) $PdAr_4TBP$'s were prepared by oxidation of $PdAr_4TCHPs$ with the 2-fold excess of DDQ (16 eq) in refluxed THF. During refluxing, the color changed from dark-red to deep-green. The solvent was evaporated, the residue was diluted with CH_2Cl_2, washed with 10% aq Na_2SO_3, water and brine. The organic phase was dried over Na_2SO_4 and concentrated in vacuum. The residue was purified by chromatography on silica gel (eluent CH_2Cl_2). The first dark-green fraction was collected, concentrated in vacuum to give 85-90% of PdAr4TBP's as blue-green powder.

2.4) Peripheral ester groups of Pd tetrakis-(3,5-dibutoxycarbonylphenyl)-tetrabenzoporphyrin (PdAr4TBP) were hydrolyzed using 10-fold excess of KOH in 1% (v/v) water/THF at rt. The solvent was decanted from the precipitate of the formed potassium salts of carboxylic acid derivatives of PdAr4TBP's. The solids were dissolved in water, stirred for additional 2 h, acidified with conc. HCl to pH~4-5. The obtained precipitate was isolated by centrifugation, washed with water and dried in vacuum.

Protecting dendrons. Each individual branch of the dendrimer is called *dendron*. Protecting dendrons are constructed from aryl-glycine (AG) building blocks. AG dendrons can be easily synthesized from inexpensive starting materials and isolated in high purity and yield by chromatography-free methods. Synthesis of generation 2 (G2) AG-dendron consists of the following steps:

2.4) Synthesis of the building blocks, Boc-protected 3,5-dicarboxyphenyl glycineamide and 3,5-dibutoxycarbonylphenyl glycineamide, using of the Fischer haloacyl halide method.

2.5) Coupling of the building blocks using CDMT/NMM peptide coupling chemistry.

2.6) Deprotecting the focal amino group by stirring the solution of the G2 dendron in TFA for 2 h at R.T.

2.7) For assembly of the higher generation dendrons, coupling/deprotection steps are repeated.

Dendrimer assembly is achieved by coupling of the deprotected dendrons to PdAr4TBP (or other porphyrin) using the HBTU/DIPEA coupling chemistry.

2.8) First, the Boc-protective groups are removed from the dendron. For example, Boc-protected G3 dendron (0.58 g) was dissolved in TFA (15 ml), and the mixture was left to react at R.T. for 2h. The acid was removed by rotary evaporation, yielding the dendron as the TFA-salt. The residue was dissolved in dry NMP (10 ml) and a few drops of DIPEA were added, to quench the traces of TFA.

2.9) Before starting the coupling reaction, it is critical to completely dissolve the porphyrin. For example, Pt tetracarboxyphenylporphyrin (0.061 g) was dissolved in dry NMP (65 ml) under heating at 140°C for 10 min, under nitrogen flow.

2.10) The solution was cooled down to room temperature, HBTU (0.211 g) was added, and the mixture was stirred for 5 min.

2.11) DIPEA (0.35 ml) was added to the mixture in one portion, immediately followed by addition of the solution of TFA-salt of the dendron in NMP (see 2.8), and the mixture was left overnight under stirring.

2.12) The mixture was poured into 3% aq. NaCl (350 ml), and the resulting precipitate was collected by centrifugation and washed with water, MeOH, and Et_2O by repetitive suspension/centrifugation to yield the target porphyrin-dendrimer.

2.13) To hydrolyze the peripheral ester groups, the dendrimer was first treated with NMe_4OH (~ 5 mM) in DMSO/MeOH over 20-60 min period, followed by the complete solvent removal. This procedure allowed us to generate dendrimers with sufficient number of carboxylate groups to give aqueous solubility. To complete the hydrolysis, the dendrimer was treated with 0.1N aqueous NaOH (overnight). As a result, pure porphyrin-dendrimer carboxylic acids could be isolated in 80-95% yields.

Modification of the dendrimer periphery. At this point, starting from the same porphyrin-dendrimer, either one- or two-photon probes can be synthesized. In the first case, all the peripheral carboxyl groups are modified with PEG units - a methodology known as *PEGylation*. In the latter case, several carboxyl groups are first coupled with two-photon antenna chromophores (Coumarin-343) modified with ethylenediamine (EDA) linkers, followed by PEGylation of the remaining carboxyls [6]. Dendrimers were PEGylated using monomethoxyoligoethyleneglycolamine (m-PEG-NH_2, Av. MW 1000) using HBTU/DIPEA coupling chemistry.

2.14) To a solution of the dendron in NMP (or DMF) (1-10 mM) 1.25-fold excess of HBTU was added, and the reaction mixture was stirred at R.T. for 10 min. 6.25-Fold excess of DIPEA was added, followed by the immediate addition of 1.25-fold excess of m-PEG-NH2. The reaction mixture was stirred for 2 days at rt. Diethyl ether was added to the reaction mixture, the formed precipitate was separated by centrifugation and re-precipitated from THF by addition of diethyl ether.

2.15) Final purification was achieved with size-exclusion chromatography by the collecting of the first front phase. PEGylated porphyrin-dendrimer was dissolved in a small amount of THF (~15 ml) and loaded on the top of a chromatographic column filled with polystyrene SEC phase (Biorad, Biobeads S-X1). The column was eluted with pure THF, and the dark colored band moving with the front was collected.

2.16) THF was removed on a rotary evaporator, and the remaining material was dried in vacuum.

3. Probe characterization

Photophysical characterization includes measurements of the probe's absorption and emission spectra, phosphorescence lifetimes τ_0 and Stern-Volmer oxygen quenching constants k_q under physiological conditions.

3.1) The absorption and emission spectra are obtained under ambient conditions using standard instrumentation (spectrophotometer and steady fluorometer). ~1 μM solutions of the probe is used.

3.2) Next, the Stern-Volmer calibration plot is obtained. This is the most important measurement, because it allows us to relate the lifetime of the probe to the oxygen concentration.

3.3) A solution of the probe in PBS buffer (pH 7.2) is placed in a special cylindrical cuvette, which is positioned inside the temperature controlled chamber, located inside a light-impermeable cage with ports for excitation and emission optical fibers. The fibers are brought into close contact with the cuvette inside the chamber in the right-angle geometry.

3.4) The cuvette is closed with a stopper, into which a highly sensitive Clark-type oxygen electrode (CK-oxygen electrode) is inserted. The stopper also contains two needle ports for inlet and outlet of argon. The electrode is immersed into the solution, whereas the needles merely provide for the flow of gas flow above the solution surface. At first, argon is not connected to the inlet.

3.5) The temperature is set to the desired value (typically 36-37°C) and solution is left under stirring for equilibration.

3.6) The excitation fiber is connected to the excitation port of a digital phosphorometer, operated by a PC. The light source in the phosphorometer is a high-power LED, whose output is controlled by a 333 kHz D/A board. The emission fiber is connected to another optical port of the phosphorometer, which is coupled to a highly infrared-sensitive APD (avalanche photodiode). The output of the diode is amplified and fed into the A/D channel of the same control board, which allows synchronization between the excitation and emission channels.

3.7) The control software allows generation of excitation pulses of any desired length (e.g. 5 or 10 ms), followed by collection of phosphorescence decay during any arbitrary chosen period (typically 2-3 ms). Time required for a single lifetime measurement is typically 0.5-1 s, however, measurements can be obtained as rapidly as 10-20 per second.

3.8) The output of the oxygen electrode is amplified and directed into another A/D board on the same PC. This is a low frequency board (1 kHz max), which is used to record the electrode current at selected time periods. The program for recording the electrode data runs simultaneously with the phosphorometer software.

3.9) Once the solution temperature is equilibrated, both the phosphorometer and the electrode programs are initialized at the same time to perform measurements every 10 s. Their outputs are logged synchronously into two separate files. After that, argon is connected to the inlet port on the cuvette stopper.

3.10) As argon flows over the surface of the stirred solution, it gradually replaces oxygen. This results in a decrease of the electrode current and an increase in the phosphorescence lifetime, which is measured by the phosphorometer. Usually, oxygen is displaced form the solution entirely after about 2 h, during which time the data are fully automatically logged into files.

3.11) After the end of the titration run, the electrode data and the phosphorescence lifetimes are imported into a standard analysis program (e.g. Miocrocal Origin). The electrode current depends linearly on oxygen concentration, and for the electrode used it is practically zero at zero

oxygen. At atmospheric pressure, pO_2 is known (about 150 mm Hg). Thus, the electrode data can be directly converted to the oxygen scale, and the plot of inverse phosphorescence lifetime vs. pO_2 can be constructed. This plot is fitted with a straight line using the least-squares method to give the oxygen quenching constant kq as its slope. Phosphorescence lifetime t0 is obtained either from the same fit (as the inverse of the intercept) or directly from the measurement at zero-oxygen.

3.12) If required, the titration is repeated using a solution of the probe in the presence of albumin - a protein present in the blood plasma in high concentrations, - in order to emulate the conditions met in the real experimental system (blood of an animal *in vivo*). The obtain Stern-Volmer plots should be identical if the dendrimer is protecting the probe well and PEG groups isolate the probe from contacts with albumin. Otherwise, the probe and the proteins in the blood will interact, and that will lead to an altered Stern-Volmer plot, causing ambiguity in oxygen measurements.

Thus obtained calibration constants are used in imaging experiments where the oxygen concentrations are *a priori* unknown.

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