POTENTIAL USAGE OF LIPOSOME-ENCAPSULATED PHOSPHOR FOR IN VIVO IMAGING OF TISSUE OXYGENATION

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ABSTRACT

Oxygen-dependent quenching of phosphorescence can provide a quantitative measurement with high temporal resolution of tissue oxygenation in vivo. It is a real-time optical means for prognosis of diseases where the oxygen concentration is essential. Phosphorescence quenching is a non-invasive methodology, otherwise no more than minimally invasive as the phosphor is necessarily introduced into vasculature prior to the measurement. Oxyphor R2, a dendritic phosphor with two-layer of glutamates, is a suitable phosphor for oxygen measurements owing to its high water solubility. We used a frequency-domain, phase modulation based instrument to calibrate Oxyphor R2. The acquired quenching constant (k_Q) and lifetime at zero oxygen (τ^0) were consistent with those calibrated from conventional time-domain instrument. Administered with Oxyphor R2, the rat hepatic oxygen distributions were imaged throughout the course of ischemia and reperfusion. After 5 min ischemia and subsequent 20 min reperfusion, distinct ischemic areas on the hepatic tissue were observed. In order to extend the application of in vivo oxygen imaging using phosphorescence quenching by minimizing the possible immunoresponse induced by phosphor, we are the first to co-synthesize the dipalmitoylphosphatidylglycerol (DPPG)-rich liposome with Oxyphor R2 to generate the liposome-encapsulated Oxyphor R2. Its calibration using the frequency domain measurement displayed higher quenching constant and shorter lifetime at zero oxygen ($k_Q = 1186 \text{ mmHg}^{-1}\text{sec}^{-1}$; $\tau^0 = 150 \text{ } \mu\text{sec}$) comparing to original Oxyphor R2 ($k_Q = 438 \text{ mmHg}^{-1}\text{sec}^{-1}$; $\tau^0 = 630 \text{ } \mu\text{sec}$). It implicated that the liposome-encapsulated Oxyphor R2 designed to neutralize the immunoresponse could be further applied to measure the tissue oxygenation in vivo, especially those with low oxygen concentration such as tumor.

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1. INTRODUCTION

Functional tissue imaging has been recognized as a pivotal tool for clinical assessment due to its ability to provide both morphological and metabolic information that pertinent to the specific disease

prognosis. Understanding that oxygen is an essential metabolite and whose disturbance is underlying to various pathophysiological states, a functional tissue imaging methodology based on oxygen-dependent quenching of phosphorescence has been continuously developed and validated as an effective method to non-invasively measure the oxygen concentration in biological systems [1-2]. It can provide rapid and accurate measurements of tissue oxygen levels or two dimensional imaging of oxygen map through thickness of tissue [3-4]. The recent development of this method has been advanced to non-invasively assess the *in vivo* oxygen distribution in tissue by using a multi-frequency system to perform the time-resolved phosphorescence analysis [5]. The capability of determining the distribution of oxygen (oxygen histogram) in the microvasculature of tissue was further demonstrated with measurements of the oxygenation of subcutaneous Q7 tumors on Buffalo rat models [6].

Based on the principle of the phosphorescence quenching [1, 7], a standard measurement is proceeded with injection of phosphorescence compound into the blood vessel and followed by using a bifurcated light guide to conduct light from the excitation source to the surface of the tissue and to return the phosphorescence to the detector [5]. The oxygen concentration can be further determined by incorporating the measured phosphorescence lifetime into a well-defined relationship called the Stern-Volmer equation:

$$PO_2 = (1/k_Q) [(1/(\tau)) - (1/(\tau^\circ))] \quad (1)$$

, where τ° is the lifetime in the absence of oxygen, τ is the lifetime at an oxygen pressure PO_2 , and k_Q is a constant describing the quenching collisions between the phosphor molecules in the triplet state and molecular oxygen [8].

Generally there are two approaches for the instruments operating phosphorescence lifetime measurements. One is in frequency domain and the other is in time domain. The measurement in frequency domain is preferred to that in time domain for a variety of reasons including increase of signal to noise ratio [9]. Measurement of the fluorescence or phosphorescence lifetime in the frequency domain is generally called the harmonic or phase modulation method. The principle of using phase modulation method for the measurement of fluorescence or phosphorescence lifetime has been described in detail previously [10]. Nevertheless, for taking the advantage of current technology advances in fast gated, intensified CCD camera, the time domain data acquisition and analysis are prevailing in the implementation of fluorescence and phosphorescence lifetime imaging systems.

As a method for measuring oxygen, phosphorescence quenching has several desirable characteristics. First, it is a noninvasive optical method. When phosphors are dissolved in the blood, phosphorescence can be used for oxygen measurements in living tissue. Second, the response time is very short within milliseconds, and thus the measurements can be made in real time and are capable of following rapid transients in oxygen pressure. Third, if the phosphorescence quenching is well behaved, the dependence of phosphorescence lifetime on oxygen pressure follows a well-defined physical relationship, which can readily be expressed in a linear equation. This greatly facilitates data analysis. Fourth, in biological systems, phosphorescence quenching is highly specific for oxygen.

A fifth, and very valuable, characteristic of this method is that the calibration is dependent only on the phosphor and its molecular environment. When the environmental parameters are held constant, the calibration is absolute. This means that, unlike oxygen electrodes and most of the other available methods for measuring oxygen, once the calibration constants are determined they can be generally applied, independent of the operator or, within limits, the preparative procedures [9]. The calibration constants including the quenching constant and lifetime in the absence of oxygen need to be determined for physiological ranges of pH, temperature and ionic strength. They can therefore be used by anyone wishing to make oxygen measurements under similar experimental conditions, including measurements of the oxygen pressure in blood *in vivo*. These values may also be used for verification of the performance of the phosphorescence measuring device and/or instrument settings [8].

For measuring oxygen concentration using phosphorescence quenching, one of the most readily available and easily used phosphors is Pd-meso-teta (4-carboxyphenyl) porphyrin (PdTCPP). However, PdTCPP has to tightly bind to albumin prior to measurements in order to increase its water solubility [11]. To further improve the water solubility, a new class of porphyrins, based on dendritic construction of surround environment of the porphyrin core has been synthesized [12-13]. A two-layer of glutamates modified PdTCPP (dendritic Oxyphor R2) provides high water solubility, no observed toxicity, good protection from chemically active components of tissue and appropriate size for excretion through kidney. In the present study, we calibrated Oxyphor R2 for oxygen measurement in frequency domain and validated all the quenching parameters consistent with those reported in previous study [5, 11]. Furthermore, we demonstrated the applicability of Oxyphor R2 for *in vivo* measurement by acquiring the quantitative

imaging of oxygen distribution in rat liver under ischemia and reperfusion with our implemented imaging system as stated in [8].

Except the necessary administration of phosphor molecules *prior to* the measurement, oxygen-dependent quenching of phosphorescence has been otherwise considered as a non-invasive optical tissue imaging method. Therefore, the increase of efficiency of *in vivo* oxygen measurement in terms of reducing immune response and increasing on-site delivery efficiency has to be emphasized. Biocompatible carrier, such as liposome, could be co-synthesized with phosphor molecules to generate liposome-encapsulated Pd-porphyrin to achieve these inquiries.

Liposomes have attracted considerable attention in recent years as model systems to study membrane phenomena, and also on account of potential applications as vehicles for drug delivery. For drug delivery applications, the liposomes can entrap soluble materials within the aqueous interior or alternatively hydrophobic materials can be bound to the bilayer membrane. By incorporating appropriate antibody while the formation of liposomes, it can render a target-search capability and provide the liposomes as on-site carriers for therapeutic agents. In addition, to avoid recognition by cell of the mononuclear phagocyte system (MPS) and uptake by the reticuloendothelial system (RES), several research groups have reported the increased localization of sterically stabilization liposome (PEG-liposomes) at tumor sites [14-16]. The long circulating "stealth" liposomes showed low toxicity and increased bioavailability.

In consideration of the phosphors used for oxygen measurements, it consists of two major components: optical chromophor and surrounding environment. Optical chromophor (OC) is a part of the phosphor, which is responsible for the phosphorescence. These useful properties are: (1) high absorption in the region of spectrum where natural chromophors of tissue, such as hemoglobin or myoglobin, do not absorb light, or absorb light only slightly; (2) phosphorescence with high quantum yields at room temperature (> 2%) and moderately long lifetimes (0.1-1 msec) in the absence of oxygen. Surrounding environment (SE) is a ligand or set of ligands linked to a chromophor skeleton to provide an environment with the properties desirable for the oxygen measurements. Ideally, SE forms an inert globular structure around the chromophor which isolates it from alterations in the solvent, but through which small-uncharged molecules, such as oxygen, can penetrate to allow phosphorescence quenching. While encapsulating dendritic Pd-porphyrin such as Oxyphor R2, the unilamellar phospholipid may be considered as a modified SE in a larger scale and which may impose an impeding effect on oxygen diffusion towards to OC.

In the present study, we are the first group to co-synthesize the dipalmitoylphosphatidylglycerol (DPPG)-rich liposome with Oxyphor R2 to generate the liposome-encapsulated Oxyphor R2 and for which, the *de novo* calibration of k_Q and τ° was preliminarily established as well to verify its further applicability for oxygen measurement *in vivo*.

2. METHODS AND MATERIALS

2.1 Reagents and Materials for Liposome Preparation

All inorganic chemicals and organic solvents used were reagent grade or better. Cholesterol, poly(vinylpyrrolidone) (PVP, MW 10 000), Tween-20, gelatin, Sephadex G-50, triethylamine (TEA), Trizma base (tris[hydroxymethyl]aminomethane, Tris), and *n*-octyl β -D-glucopyranoside (OG) were purchased from Sigma (St. Louis, MO). Dipalmitoylphosphatidylethanolamine (DPPE) and sulforhodamine B were purchased from Molecular Probes (Eugene, OR). Polycarbonate syringe filters of 3-, 0.4-, and 0.2- μ m pore size were purchased from Poretics (Livermore, CA), and plastic-backed nitrocellulose membranes with pore size of >3 μ m were from Schleicher and Schuell (Keene, NH). Dipalmitoylphosphatidylcholine (DPPC) and dipalmitoylphosphatidylglycerol (DPPG) were obtained from Avanti Polar Lipids (Alabaster, AL). *N*-Succinimidyl-S-acetylthioacetate (SATA), 4(4-*N*-maleimidophenyl)butyric acid hydrazide (MPBH), and *N*-ethylmaleimide (NEM) were purchased from Pierce (Rockford, IL). Carnation nonfat dry milk (NDM) powder was obtained locally.

Dye-loaded liposomes were prepared by the reversed-phase evaporation method [17]. The lipids mixture consisted of a 5:5:0.5:4 molar ratio of DPPC, cholesterol, DPPG, and DPPE-acetylthioacetate. The lipids were dissolved in 8 mL of a solvent mixture consisting of 6:6:1 volume ratios of chloroform, isopropyl ether, and methanol, followed by a 1-min sonication at 45 °C under nitrogen. Then 1.4 mL of a warmed aqueous solution of 150 mM Oxyphor R2 in TBS, adjusted to pH 7.0-7.5 with 1N NaOH, was added. After sonication of the solution for 6 min more, the organic solvent was removed by evaporating under nitrogen at 45 °C, leaving a dark purple, gel-like suspension of liposomes. An additional 2.6 mL of Oxyphor R2 dye was added, followed by another 5 min of sonication at 45 °C. Liposomes were kept warm before passing through three different pore sizes of prewarmed polycarbonate filters (3.0, 0.4, and 0.2 μ m) to produce a homogeneous suspension. The liposomes were separated from unencapsulated

Oxyphor R2 and traces of organic solvent by gel filtration on a 1.5×25 cm Sephadex G-50 column at room temperature, followed by dialysis (MWCO, 12 000-14 000) in 0.01 M Tris-HCl, 0.15 M NaCl, pH 7.0 at 4°C in the dark.

2.2 Stability Study and Characterization of Liposome

Oxyphor R2 is a phosphorescence molecule; thus, the stability (intactness) of liposomes means the maintenance of their integrity, which can be determined by measuring phosphorescence intensity before and after lysis. Almost instantaneous lysis of liposomes was observed at room temperature when a solution of *n*-octyl β -D-glucopyranoside was added; total lysis of the liposomes was achieved by addition of OG to a final concentration of 50 mM. For these phosphorescence tests, the Oxyphor R2 dye was excited at 525 nm, and the phosphorescent emission intensity measurements were made at wavelength 596 nm. Temperature effects on liposome stability were conducted by adding 10 μ L of liposome solution to 3 mL of osmotically balanced TBS, pH 7.0, in a test tube preheated to the required temperature in a water bath. The liposomes were incubated for 5 min before measurement of phosphorescence intensity.

The diameter of the liposomes was measured with a Coulter LS particle size analyzer (Coulter Corp., Miami, FL) using the manufacturer's method. Liposomes were visualized by transmission electron microscopy of negatively stained preparations to check the integrity and size of the liposomes.

2.3 In Vitro Calibration of Oxyphor R2 and Liposome-encapsulated Oxyphor R2

As mentioned, the essence of the method of oxygen-dependent quenching of phosphorescence is the calibration, which depends only on the phosphor and its molecular environment. In order to precisely calibrate the quenching constants and lifetimes at zero oxygen, a special instrument and methodology were designed as described in [11]. In brief, Oxyphor R2 (Oxygen Enterprises, Philadelphia, PA) or liposome-encapsulated Oxyphor R2 was dissolved in a pH 7.4 buffer solution containing 20 mM MOPS, 10 mM KH_2PO_4 , 0.12 M NaCl, and 2% by weight of bovine serum albumin to the final concentration of 10 μ M. A round glass vial containing 2 ml of phosphor solution to be calibrated was sealed with a stopper pad through which a polarographic oxygen electrode (World Precision Instrumentation) was inserted. The glass vial was thermostatted at 38°C and stirred with a magnetic stirring bar. Oxygen of the solution was slowly removed by passing a stream of purified argon gas. The phosphorescence signal was conducted through a

bifurcated light guide that was arranged in a way for better representation of the lifetime near the tip area of the oxygen electrode. This can minimize the discrepancy caused by the oxygen gradient between the solution surface to the tip of the electrode. A frequency domain phosphorescence lifetime measuring system (PMOD 5000; Oxygen Enterprises, Philadelphia, PA) was used for the simultaneous measurement of the phosphorescence lifetime.

2.4 Phosphorescence Imaging System for Oxygen Measurements

The implementation of phosphorescence lifetime imaging system and the procedure to calculate the pixel by pixel oxygen concentration have been detailed described in our previous publication [8]. The specific delay time sequence selected for Oxyphor R2 was: 20, 40, 80, 160, 300, 480, 760, and 2500 μ sec.

2.5 Animal Preparation

All animal procedures were in accordance with the National Health Research Institutes guidelines for the care and use of laboratory animals. Male Sprague-Dawley rats (260-350 gm) were deprived of food and had free access to water for 12 hours before the experiments. Rats were anesthetized with urethane (1.2 g/kg body weight i.p.) and allowed to breathe spontaneously. The body temperature was maintained at 37°C with a heating plate. Polyethylene catheters were inserted into the femoral artery for intravenous administration (bolus) of Oxyphor R2 (50 mg/kg body wt).

After a transverse upper abdominal laparotomy, the liver was mobilized and isolated by dividing all of its attachments with negligible bleeding. Temporal ischemia was introduced with the procedure similar to the portal triad clamping model (PTC model) that used total ligation of the vessels (hepatic artery, portal vein, and bile duct) in the hepatoduodenal ligament. The hepatic oxygen distribution was imaged after undergone 5 min ischemia and 20 min reperfusion.

3. RESULTS

3.1 Characteristics of Oxyphor R2

Oxyphor R2 was a Pd-meso-teta (4-carboxyphenyl) porphyrin (PdTCPP)-dendrimer (fig. 1a). It was synthesized with two layers of glutamate modified on the core Pd-porphyrin, PdTCPP. The 16 carboxyl groups located on the outer layer of the dendritic Oxyphor R2 render total net 16 negative charges at near neutral pH which not only provide high water solubility but also prevent the molecule itself

"leaking" from the blood vessels. These properties make Oxyphor R2 a prominent phosphor for measuring tissue oxygenation which represents the oxygen concentrations distributing in the tissue vascular system. The spectral analysis indicated that Oxyphor R2 has two absorption maxima at 421 nm (Soret-band) and 525 nm (Q-band), and a phosphorescence emission at 695 nm (fig. 1b). The amount needed for oxygen measurements is in the range of 15-60 mg/kg body wt.

3.2 Hepatic Oxygen Distribution under Ischemia and Reperfusion

The change of hepatic oxygen distribution under ischemia and reperfusion was monitored using phosphorescence imaging system. The imaged area of rat liver was first taken using reflected room light (fig. 2a) whose morphological features could be co-localized with later imaged oxygen maps. The hepatic oxygenation was heterogeneously distributed over the range of 30 to 55 mmHg before the engagement of PTC ligation (fig. 2b). In contrast, after the course of 5 min ischemia with PTC ligation and subsequent 20 min reperfusion, the hepatic oxygen distribution showed the decrease of oxygen pressures to the range of 20 to 40 mmHg and the distinct border zone of ischemic tissue (fig. 2c). All the oxygen maps were calculated from acquiring a series of intensity images with a specific delay time sequence selected for Oxyphor R2: 20 μ sec, 40 μ sec, 80 μ sec, 160 μ sec, 300 μ sec, 480 μ sec, 760 μ sec, and 2500 μ sec. The ischemic area of the liver was contoured with prominent phosphorescence intensity after the 300 (sec intensifier gating delay, from which the oxygen pressure was calculated around 20 mmHg (Data not shown). The image collected after a delay of 2500 (sec was used as the background and subtracted from each of the other phosphorescence images to prevent any light intensity arisen from non-phosphorescence sources into the integrated images.

3.3 Characteristics of Liposome-encapsulated Oxyphor R2

The characteristics of liposomes are listed in table 1. With liposomes of 346-nm diameter, it is possible to calculate that the average volume of a single liposome is $2.17 \times 10^{-11} \mu\text{L}$ and the volume entrapped (assuming a bilayer thickness of 4 nm) is $2.02 \times 10^{-11} \mu\text{L}$. Assuming the dye concentration inside of the liposomes was equal to the original dye solution used, and comparing the fluorescence of lysed liposomes to that of standard Oxyphor R2 solutions, it is possible to calculate that there were $\sim 4.79 \times 10^{10}$ liposomes/mL and that each liposome contained $\sim 3.1 \times 10^5$ molecules of dye.

Fig 1. The chemical structure of Oxyphor R2, a Pd-meso-tetra (4-carboxyphenyl) porphyrin (PdTCPP)-dendrimer (a). It was synthesized with two layers of glutamate modified on the core Pd-porphyrin, PdTCPP. The spectral analysis indicated that Oxyphor R2 has two absorption maxima at 421 nm (Soret-band) and 525 nm (Q-band), and a phosphorescence emission at 695 nm (b).

In order to prevent the extravasation of liposome-encapsulated Oxyphor R2 from the vasculature, the liposomal lipid bilayer was carefully synthesized with a composition rich in dipalmitoylphosphatidylglycerol (DPPG), as described in materials and methods, so that to maintain the overall negative charge on the outer surface of liposome (fig. 3a). The spectral analysis demonstrated that the liposome-encapsulated Oxyphor R2 conserved the optical property of Oxyphor R2. It

Fig 2. The change of hepatic oxygen distribution under ischemia and reperfusion was monitored using phosphorescence imaging system. (a) The imaged area of rat liver was first taken using reflected room light whose morphological features could be co-localized with later imaged oxygen maps. (b) The hepatic oxygenation was heterogeneously distributed over the range of 30 to 55 mmHg before the engagement of PTC ligation. (c) After the course of 5 min ischemia with PTC ligation and subsequent 20 min reperfusion, the hepatic oxygen distribution showed the decrease of oxygen pressures to the range of 20 to 40 mmHg and the distinct border zone of ischemic tissue.

Table 1. Characteristics of the Liposomes

mean diameter (nm)	346 nm
volume of liposome (μ L)	2.17×10^{-11}
entrap volume of liposome (mL)	2.02×10^{-11}
liposome concentration (number/mL)	4.79×10^{10}
Oxyphor R2 concentration (mM)	26
Oxyphor R2 molecules per liposome	3.1×10^5

has two absorption maxima at 418 nm (Soret-band) and 525 nm (Q-band), and a phosphorescence emission at 706 nm (fig. 3b). This observation further facilitates using liposome as a carrier for Oxyphor R2 for *in vivo* oxygen measurements as which would not disturb this dendrimer-porphyrin's optical characteristics.

3.4 Calibrations of Oxyphor R2 and Liposome-encapsulated Oxyphor R2

Phosphorescence quenching parameters for Oxyphor R2 with 2% bovine serum albumin with and without liposome encapsulation at pH 7.4, 38°C were calibrated. The reciprocal of measured phosphorescence lifetimes versus corresponding oxygen pressure measured by polarographic oxygen electrode was shown in figure 4. According to Stern-Volmer equation, the slope of each plot represents its quenching constant k_Q . The lifetime at zero oxygen τ° could be acquired from the intercept of the plot. As indicated in figure 4, the liposome-encapsulated Oxyphor R2 displayed higher quenching constant and shorter lifetime at zero oxygen ($k_Q = 1186 \text{ mmHg}^{-1}\text{sec}^{-1}$; $\tau^\circ = 150 \mu\text{sec}$) comparing to Oxyphor R2 ($k_Q = 438 \text{ mmHg}^{-1}\text{sec}^{-1}$; $\tau^\circ = 630 \mu\text{sec}$).

4. DISCUSSION

We currently use phosphorescence probes that bind tightly to albumin. In *in vitro* experiments, albumin is added in molecular excess over the phosphor whereas in *in vivo* experiments there is an excess of albumin in the blood. The albumin-binding site provides a well-defined physical environment with a controlled access by oxygen. Since the environment of the phosphors independent of the medium (other than albumin), calibration of k_Q and τ° *in vitro* are fully valid for studies *in vivo*. In addition, as long as each is bound to albumin, in the concentration range of phosphors used in our studies there is no significant "self quenching" due to energy transfer from the triplet state to ground state phosphor molecules. To use liposome as Oxyphor R2 carrier, the liposomal lipid

Fig 3. (a) Oxyphor R2 was encapsulated with liposome, whose lipid bilayer was carefully synthesized with a composition rich in dipalmitoylphosphatidylglycerol (DPPG) so to maintain the overall negative charge on the outer surface. (b) The spectral analysis indicated that the liposome-encapsulated Oxyphor R2 conserved the optical property of Oxyphor R2. It has two absorption maxima at 418 nm (Soret-band) and 525 nm (Q-band), and a phosphorescence emission at 706 nm.

bilayer was synthesized with a composition rich in dipalmitoylphosphatidylglycerol (DPPG) and thereby increasing the overall negative charge distribution on the surface of liposome to minimize the "leakage" of liposomes from blood vessels during their circulation. However, the bulk of negative charge would result in

loose association of liposome-encapsulated Oxyphor R2 to albumin, which is composed of highly surface negative charges at pH 7.4 as well. This may contribute at least in part towards disturbance of the well-defined physical environment provided by albumin and result in facilitated access by oxygen to incur phosphorescence quenching. The increase rate of collision between oxygen and Oxyphor R2 should engender the higher quenching constant k_Q of liposome-encapsulated Oxyphor R2 ($1186 \text{ mmHg}^{-1}\text{sec}^{-1}$ to $438 \text{ mmHg}^{-1}\text{sec}^{-1}$). Furthermore, the concentrated Oxyphor R2 confined in the liposomal internal compartment without being associated with albumin, the probability of self-quenching would significantly increase to which the much shorter τ was possibly attributed ($150 \text{ } \mu\text{sec}$ to $630 \text{ } \mu\text{sec}$). In order to increase the dynamic range of phosphorescence lifetime measurements, the high quenching constant k_Q of liposome-encapsulated Oxyphor R2 would be reduced by modifying the liposomal surface with polyethylene glycol (PEG). With PEG conjugation, not only for the purpose of diminishing immune response [15-16], it could also impede oxygen diffusion in terms of dramatically increasing the surface steric effect of liposome, and thereby decrease the quenching constant.

However, while applying it to biological systems, the oxygen measurements have been limited to the surface layer of the tissue because the absorption bands of the Pd-meso-tetra-(4-carboxyphenyl)-porphyrin-dendrimer such as Oxyphor R2, were all at wavelengths less than about 540 nm. At these wavelengths, absorption of the excitation light by chromophores in the tissue, notably hemoglobin, myoglobin, and cytochrome, limits the depths in tissue to which the excitation light can penetrate (and thereby phosphorescence measurements) to about 1 mm or less. The emitted phosphorescence, in contrast, has a maximum at 695 nm, where the tissue chromophores absorb much less, and can penetrate much greater thicknesses of tissue. Vinogradov and Wilson [19-20] have reported synthesis of a group of oxygen sensitive phosphors (tetrabenzoporphyrins) with absorption maxima near 636 nm and phosphorescence maxima near 800 nm. The experiments in which a water soluble derivative of Pd-tetrabenzoporphyrin was used to determine the oxygen distribution in the vasculature of tumor bearing mice [4, 11] and in the surface and deep areas of the kidney of rats [18] have established that, by using near infra-red phosphors, it is possible to image tissue oxygen distribution through centimeter thicknesses of tissue (such as through the abdomen of an adult mouse). Thus, phosphorescence quenching can now be used both to detect the presence of small regions of hypoxia (such as subcutaneous EMT-6 tumors) present in much larger volumes of normal

Fig 4. Calibrations of Oxyphor R2 with 2% bovine serum albumin with and without liposome encapsulation at pH 7.4, 38°C. The $(1/\tau)$ vs. PO_2 delineated Stern-Volmer equation, in which the slope of each plot represented its quenching constant k_Q . The lifetime at zero oxygen τ^0 was acquired from the intercept of the plot. The liposome-encapsulated Oxyphor R2 displayed higher quenching constant and shorter lifetime at zero oxygen ($k_Q = 1186 \text{ mmHg}^{-1}\text{sec}^{-1}$; $\tau^0 = 150 \mu\text{sec}$) comparing to Oxyphor R2 ($k_Q = 438 \text{ mmHg}^{-1}\text{sec}^{-1}$; $\tau^0 = 630 \mu\text{sec}$).

tissue and to quantify the oxygen pressure in such hypoxic tissue volumes. Further design of using liposome to encapsulate the Pd-tetrabenzoporphyrin derivatives that both absorb and phosphoresce at the wavelengths in the near-infrared region should greatly increase the efficiency of using phosphorescence probes for *in vivo* oxygen measurement, i.e., with minimal amount of probes administered but to acquire high resolution of quantitative oxygen distribution imaging *in vivo*. It would take advantages both from liposome in terms of reducing immune response and increasing on-site delivery efficiency, and from the Pd-tetrabenzoporphyrin derivatives that possess better tissue penetration depth due to their characteristics of near-infrared phosphorescence.

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