Poly(ϵ -caprolactone)-containing graft copolymers for ratiometric extracellular oxygen sensing

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Abstract:

A graft copolymer composed of poly(ϵ -caprolactone) as a hydrophobic chain and poly(*N*-(2-hydroxypropyl)methacrylamide as a hydrophilic segment with an internal reference probe for oxygen sensing was prepared. Highly efficient platinum(II)meso-tetra(pentafluorophenyl)porphine (PtTFPP) was used as a typical oxygen probe. Although PtTFPP was highly efficient, it is hydrophobic, limiting its direct application for oxygen sensing in biological conditions. By using the amphiphilic graft copolymer, PtTFPP was successfully incorporated into the micelles formed by this polymer to not only enable the application of PtTFPP for biosensing in medium but also retain its high efficiency. The quantum efficiency of PtTFPP in the micelle solution under nitrogen can reach as high as 0.20. The PtTFPP-containing micelles were tested for high throughput cell respiration and bacteria detection. The influence of an antibiotic, antimycin as a representative example, on cell respiration was also studied. For achieving high accurate sensing, an internal built-in

reference probe with aggregation induced emission properties was also polymerized in the graft copolymer. This study demonstrated that the graft polymer approach is a good way to enable highly efficient and hydrophobic oxygen probes for biosensing.

Keywords:

Oxygen sensor, ratiometric sensor, graft copolymer, oxygen consumption, micelles

1. Introduction:

Oxygen is a critical element for environment, ocean, agriculture, industry, and health. In the biological field, oxygen is one of the key substrates in aerobic cells and organisms, playing a pivotal role in many core physiological and developmental cellular processes including energy production, energy homeostasis, and biomass production. As an electron acceptor, oxygen is vital for the function of the electron transfer chain (ETC) in mitochondria and ATP production. Aberrations in oxygen availability and/or utilization are known to lead to many pathological and diseased states and, thus, represent an important target for clinical and basic research. For instance, it has been demonstrated that low oxygen availability (hypoxia) can render malignant cells more metastatic and resistant to drugs via direct and indirect effects on gene expression regulation [1-3]. The metabolic switch from oxidative phosphorylation to aerobic glycolysis in cancer cells – the Warburg effect – is one of the emerging hallmarks of cancer [4, 5]. Oxygenation has been shown to be a major determinant in tissue survival and regeneration in cardiovascular ischemia and reperfusion injury [6, 7]. Hypoxia has been demonstrated to be an important factor in various diseases including Alzheimer's [8, 9], inflammation [9, 10], heart disease [9, 11], stroke [9, 11-13], chronic lung disease [9, 14], and cancer [9, 15, 16], which together are responsible for 60% of deaths in the

United States [9]. Therefore, the ability to measure cellular oxygen in real time is important for a better understanding of cell oxygenation dynamics, mitochondrial activity, and energy production and biomass synthesis homeostasis. Besides the above functions, cell respiration is related with cell viability, therefore through cell respiration measurement, it is also feasible to achieve high-throughput drug screening [17].

Various oxygen sensors in the formats of liquid, fiber-optics, hydrogels, sol-gels, polymer films, micro-/nano-particles were developed and some state-of-the-art review articles were published [18, 19]. Some advanced and commercialized instruments based on optical oxygen sensors have been developed for oxygen measurements and cell metabolism understanding. For example, Seahorse Bioscience has developed its XF96 metabolism instrument for oxygen respiration and extracellular acidification measurements [20]. The sensors in XF96 instrument belong to a kind of polymeric gel-based sensors. For measuring cell respiration and acidification, Seahorse's XF instrument uses a specific chip design to achieve its goal for high throughput analysis of extracellular pH and oxygen measurements under their sophisticated and advanced instrument. For achieving high-throughput analysis, if the materials can be used easily in many common instruments, the materials may benefit many researchers and researches. Thus if the probes/sensors can be dissolved in or suspended in cell culture medium, these materials might be a class of ideal materials to achieve the easy use and also for high throughput applications. For simplification, we named such kind of probes/sensors as liquid format probes/sensors. Many liquid oxygen probes/sensors by using organic compounds, macromolecules, and/or nanoparticles [21-33] were developed. Among these probes/sensors, although many are for intracellular oxygen measurements [22-24, 30, 31,

34-37] ; some liquid sensors were demonstrated to be extracellular sensors and were used for cell respiration and mitochondrial activity measurements [21, 28, 29]. Papkovsky et al. [21, 22] conjugated isocyanated-containing platinum(II)-coproporphyrin (PtCP) with bovine serum albumin (BSA), mono-amino polyethylene glycol (amino-PEG) and amino-dextran to form macromolecular probes. Authors used metal porphyrins (PtCPK, PtCP, PdCPK, PtTCPP) [21, 22] as hydrophilic oxygen probes. These materials showed extremely low quantum efficiencies as low as in the range of 0.001 to 0.0095. Although these macromolecular oxygen sensors and particle sensors were cell impermeable to *E. Coli* [21] and ideal for extracellular sensing, the low quantum efficiency is a weakness for biosensing and bioimaging. For achieving high quantum efficiency and endow some new features for oxygen sensors, Vinogradov et. al. presented a few series of oxygen sensors with dendrimer shapes [29, 31, 32]. The core of dendrimers is a platinum porphyrin; the shell of the dendrimers is hydrophobic. For these dendrimers, phosphorescent metalloporphyrins are encapsulated inside hydrophobic dendrimers, which form protecting shells by enveloping the chromophores to control oxygen diffusion to the excited triplet states for high sensitivity. Peripheral PEGylation or carboxylation of the dendrimers ensures high aqueous solubility and prevents interactions of the probes with biological macromolecules. As a result, quantum efficiencies of the porphyrin-containing dendrimers are around 0.017 to 0.073 [31]. However, the synthesis of dendrimer is normally time consuming and the purification is uneasy. We have used an amorphous block copolymer approach by using nanostructured micelles to encapsulate a highly efficient however extremely hydrophobic oxygen probe of PtTFPP to enable the application of PtTFPP in aqueous solution with high efficiency of form 0.107 to

0.111 [23] by using a poly(ϵ -caprolactone)-*block*-poly(ethylene glycol) block copolymer. Borisov et. al. reported an incorporation of platinum(II)meso-tetra-(pentafluorophenyl)porphine (PtTFPP) in polystyrene-*block*-polyvinylpyrrolidone beads and studied the oxygen responses in aqueous solutions [28]. The authors demonstrated that the beads with an average diameter of approximately 250 nm were impermeable for *E. coli* cells. No further results were reported by using these beads for biostudies.

On the other hand, recently, a new category of fluorophores with aggregation-induced emission (AIE) properties has been developed [38]. AIE fluorophores have been shown to have high fluorescence quantum yields in the aggregate states. Restricted intramolecular rotation has been suggested as the possible mechanism of the AIE phenomenon, considering that intramolecular rotations can drastically affect the radiative/nonradiative recombination processes of the excited state. In the biological research fields, the AIE fluorophores have been widely explored as sensors for DNA [39], heparin [40], CO₂[41], glucose [42] and bioimaging probes [38], to list a few. Herein, we extended the AIE fluorophores as internal references for oxygen sensors to achieve ratiometric sensing. The ratiometric method is capable to alleviate the environmental influences, such as fluctuations in excitation source intensity, variance in probe concentration, and uncontrollable variations in background fluorescence, and as a result, increases the measurement accuracy [43-47].

Thus, we prepared new nanostructured liquid oxygen sensors using graft-polymer as the carriers for PtTFPP. As compared with block copolymers for achieving high quantum efficiency for oxygen sensing in aqueous solutions we used before, graft copolymers are relatively easily prepared and manipulated. Some excellent nanomaterials for quantum

incorporation and drug delivery systems were prepared by using graft copolymers [48, 49]. Herein, we prepare nanostructured oxygen sensors using graft copolymer approach. Therefore, we prepared a graft copolymer as the carrier for highly efficient PtTFPP probe to enable the application of PtTFPP for biosensing. The new graft copolymer was composed of biocompatible poly(ϵ -caprolactone) as a hydrophobic chain and poly(*N*-(2-hydroxypropyl)methacrylamide as a hydrophilic segment. For achieving ratiometric sensors for accurate bioanalysis, we used AIE fluorophores as the internal standard. We have tested these sensors' characteristics, high quantum efficiency, and applicability for extracellular cell respiration using a popular lab instrument like a plate reader for high throughput *in-situ* cellular oxygen consumption measurements. We also demonstrated the sensor is suitable for low concentrated-bacteria detection and drug screening.

2. Experiments:

2.1 Materials:

PtTFPP was purchased from Frontier Scientific (Logan, Utah), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was ordered from Sigma-Aldrich (St. Louis, MO). Stannous octoate (J & K Scientific Ltd, Shanghai), Azodiisobutyronitrile (AIBN, Aladdin, Shanghai) and methacryloyl chloride, stabilized with MEHQ (TCI, Tokyo) were commercially available from different vendors. *N*-(2-Hydroxypropyl)methacrylamide (HPMA) was synthesized according to a known procedure and our publications [50, 51], AIE fluorophore was synthesized according to our

previous publication [52] .

2.2 Instruments

UV-Vis spectra were recorded with a PerkinElmer Lambda 25 UV/Vis spectrophotometer. Fluorescence spectra were recorded with a Horiba FluoroMax-4 spectrofluorophotometer. Dynamic light scattering (DLS) measurements for micelle diameters were performed using a Horiba Nano Partica SZ-100 nano particle analyzer. The solution was passed through a 0.45 μm Nylon microfilter (VWR, Batavia, IL) to remove dust before the DLS measurements. Microplate reader (BioTeck, Winooski, VT) was used for cell growth, cell toxicity, and cell oxygen respiration measurements. Molecular weights were determined by using a Waters 1515 GPC system (Waters, Milford, MA) coupled with a RI detector.

2.3 Preparation of polymers (Scheme 1)

2.3.1 Synthesis of P1: 988 mg (8.7 mmol) of ϵ -caprolactone and 61.1 mg (0.1 mmol) **1 (AIE-OH)** were mixed in a flask and stirred for 5 min at 110 °C under N_2 atmosphere. Two drops of stannous octoate ($\text{Sn}(\text{Oct})_2$) as the catalyst were injected into the flask and the reaction was continued under stirring for overnight. The polymerization mixture was cooled down rapidly in an ice bath to obtain a solidified crude polymer. This solid was re-dissolved in 5 mL DCM and the solution was precipitated into 100 mL of cool methanol to obtain the polymer. This procedure was repeated twice for getting a pure yellow-greenish polymer **P2** of 600 mg with a yield of 71%. ^1H NMR (400MHz, CDCl_3 , δ (ppm)): 8.39 (m, br, 4H, ArH), 7.80 (d, 4H, ArH), 7.60 (d, 4H, ArH), 7.46 (br, 4H, ArH), 6.99 (d, 4H, ArH), 6.88 (d, 4H,

ArH), 4.05 (t, 186H, OCH₂-), 3.66 (br, 2H, -CH₂OH), 2.30 (t, 180H, -CH₂CO), 1.64 (m, 376H, -CH₂CH₂CH₂-), 1.37 (m, 190H, CH₃CH₂- & -CH₂CH₂CH₂-). n = 90; $M_{n(NMR)} = 10860$; $M_{n(GPC)} = 4600$, $M_{w(GPC)} = 6700$, $M_w/M_n = 1.46$.

2.3.2 Synthesis of AIE-poly(ϵ -caprolactone) methacrylate (P2): 440 mg (0.4 mmol) **P1** with 1.39 mL (20 mmol) Et₃N were dissolved in 60 mL anhydrous DCM. 0.97 mL (20 mmol) methacryloyl chloride with 20 mL anhydrous DCM were added in reaction vessel over 1 hour period in ice bath. After one night reaction at room temperature, the concentrated target product was precipitated into 100 mL of MeOH in ice bath twice to obtain pure greenish polymer of 300 mg, Yield: 68%. ¹H NMR (400MHz, CDCl₃, δ (ppm)): 8.39 (m, br, 4H, ArH), 7.80 (d, 4H, ArH), 7.60 (d, 4H, ArH), 7.46 (br, 4H, ArH), 6.99 (d, 4H, ArH), 6.88 (d, 4H, ArH), 6.10 (br, 1H, CH₂=), 5.55 (br, 1H, CH₂=), 4.05 (t, 188H, OCH₂-), 2.30 (t, 180H, -CH₂CO), 1.64 (m, 376H, -CH₂CH₂CH₂-), 1.37 (m, 193H, CH₃CH₂- & -CH₂CH₂CH₂-). n = 90; $M_{n(NMR)} = 10940$.

2.3.3 Synthesis of copolymer of P3: 500 mg (3.49 mmol) of HPMA, 220 mg (0.2 mmol) of **P2**, and 10 mg of AIBN were dissolved in 5 mL of anhydrous DMF. The solution was degassed three times through a standard freeze-thaw process and refilled with nitrogen. The monomers were polymerized at 75 °C under N₂ atmosphere for 24 hours. The final polymer was precipitated into 200 mL ether from the DMF solution to get 400 mg of **P3** with a yield of 56%. ¹H NMR (400MHz, CDCl₃, δ (ppm)): 7.02–8.14 (broad, aromatic protons), 4.00 (broad, -CH₂O-), 3.65 (broad, -CH₂NH-), 2.30 (t, -CH₂CO-), 1.58 (m, -CH₂CH₂CH₂-), 1.37 (m, CH₃CH₂- & -CH₂CH₂CH₂-), 0.90 (m, 0.92 (broad, -CH₃)). Molar ratios between HPMA and PCL were calculated to be 0.98:0.02 by ¹H NMR using the integrations at the chemical

shifts of 3.65 (the CH₂NH from HPMA segments) and 4.00 (the CH₂O parts of PCL chains).

$M_{n(\text{GPC})} = 58000$, $M_{w(\text{GPC})} = 106000$, $M_w/M_n = 1.82$.

2.4 Critical micelle concentration (CMC) determination:

CMC of **P3** was determined according our previous procedure by taking the advantage of the fluorophore's emission properties [52]; When the polymer concentration is extremely low, the polymers are in the form of unimers in the solutions. Under these conditions, the polymers did not self-assemble to form micelles. Therefore, there are no obvious aggregations of the AIE fluorophores and very weak fluorescence was observed. When the polymer concentrations are over their CMCs, the polymers will form micelles to enable the aggregations of the hydrophobic AIE segments in the micellar cores. This will result in the observation of strong fluorescence. Meanwhile, the fluorescence intensities after CMC increased with the increases of concentrations. Fluorescence intensities were plotted against the polymer concentrations in double distilled water (Supporting materials Figure S1). It was found that the CMC of the polymer **P3** is as low as 0.005 mg/mL, similar to many other block copolymers' CMCs [53-55] .

2.5 Preparation of micelles

5 mg of polymer **P3** was dissolved in 200 μ L of DMSO with PtTFPP concentration of 0.5 mg/mL to obtain a clear solution. This clear solution was then injected in to 1 mL DI water slowly under stirring. The solution was then dialyzed against DI water for about two days with a water exchange at an interval of about 12 hours to remove the organic solvent of DMSO for generating micelles in pure water. After dialysis, the solution was filtered through

0.45 µm micro-filter to remove any possible precipitated polymers or PtTFPP not in micelles or large particles formed. Concentration of PtTFPP and AIE fluorophore in micelles were measured using UV-Vis absorption spectra by referring the calibrated curves of PtTFPP and/or AIE fluorophore in a mixture of DMSO/water (90:10 by volume) mixed solution, respectively. The filtered and calibrated micelle solution in brown bottle was stored in a 4°C refrigerator for further use. It was determined that the micelles contained 0.095 mg/mL of PtTFPP (corresponding to a PtTFPP concentration of 75 µM), and the polymer **P3** concentration for the micelle stock solution was 4.8 mg/mL (corresponding to an AIE fluorophore concentration of 330 µM). The micelles remained stable in a 4°C refrigerator for at least one month without an alternation of photophysical properties and/or sizes. This solution was a stock solution for photophysical and bio-studies. Other micelles with different PtTFPP concentrations (ranging from 0.02 to 0.5 mg/mL) with a consistent AIE-polymer concentration of **P3** at ~ 4.8 mg/mL were prepared similarly for fluorescence resonance energy transfer (FRET) investigations.

2.6 Determination of quantum yields

The fluorescence quantum yields (η) of PtTFPP in **P3** micelle solutions were recorded by using PtTFPP in methylene chloride ($\eta = 0.088$) [56] excited at 390 nm and were calculated according to the following equation [57]:

$$\eta_s = \eta_r \left(\frac{A_r}{A_s} \right) \left(\frac{I_s}{I_r} \right) \left(\frac{n_s^2}{n_r^2} \right) \quad (1)$$

where (η_r) and (η_s) are the fluorescence quantum yields of standards and the samples, respectively. A_r and A_s are the absorbance of the standards and the measured samples at the

excitation wavelength, respectively. I_r and I_s are the integrated emission intensities of standards and the samples. n_r and n_s are the refractive indices of the corresponding solvents of the solutions, respectively. Refractive indexes of H_2O and CH_2Cl_2 are 1.333 and 1.424, respectively. The final value of quantum yield was obtained from an average of four measurements with different absorbance in the range between 0.03 and 0.09. The standard deviation is less than 10%.

Similarly, fluorescence quantum yields for the AIE fluorophores were obtained by comparing the integrated fluorescence spectra of the polymers in solutions to the fluorescence spectrum of quinine sulfate in 1.0 N H_2SO_4 ($\eta = 0.55$, excitation wavelength of 365 nm) with corrections of refractive index differences using equation shown above.

2.7 Oxygen sensing performance

Nitrogen and oxygen gas mixtures were used to saturate the solution to adjust the dissolved oxygen concentrations. The gas mixtures were precisely controlled with a custom-built, in-line, digital gas flow controller. All sensing measurements were carried out at atmospheric pressure, 760 mmHg or 101.3 kPa. At the air saturated condition with an oxygen partial pressure of 21.4 kPa, the dissolved oxygen concentration in water is 8.58 mg/mL at 23°C.

2.8 Response time

2.8.1 Response time measured through the bubbling with a mixture of nitrogen and oxygen gas

Response time was measured through bubbling with a mixture of nitrogen and oxygen gas. 3 mL of PtTFPP micelles was placed in a quartz cuvette. Emission measurements were taken every 3 seconds at the sensor's peak emission of 650 nm and excited at 405 nm. To vary the concentrations of dissolved oxygen in the buffers, we used a gas manifold to control gas flow through a tube and needle to bubble the buffers. Holes were installed in the cuvette cap to allow the needle into the cuvette and gas flow out. Oxygen and nitrogen flow rates were set at 50 standard cubic centimeters per minute. Measurements began with an immediate change from the oxygen flow into the buffer to 100% nitrogen, and then continued as the gas flow composition was then immediately reversed between oxygen and nitrogen.

2.8.2 Response time measured using glucose oxidase (stationary response time)

2 mL of PtTFPP micelles was placed in a quartz cuvette. Glucose was added to reach a glucose concentration of 0.25 M. Different amounts of glucose oxidases (concentration: 10 mg/mL) were added into the micelle-glucose solutions. Glucose oxidase oxidizes glucose to consume oxygen. Emission measurements were taken every 0.06 seconds at the sensor's peak emission of 650 nm and excited with 405 nm.

2.9 Cell culture of *E. Coli*

E. coli were cultured in liquid Lysogeny Broth (LB) medium from a single colony. The LB medium was made of 10 g tryptone, 5 g yeast extract and 10 g NaCl in 1 L distilled water. The cell density of *E. coli* in the culture medium was estimated by measuring the optical density at 600nm (OD600), which is linearly proportional to *E. coli* cell density when OD600

is between 0.1 and 1.0. A value of 1 indicated a cell density of 5.0×10^8 colony-forming unit per milliliter (cfu/mL), based on calibrations of our UV-vis spectrophotometer. After *E. coli* cell density of the overnight culture was determined, appropriate dilutions were made using fresh LB to achieve desired initial *E. coli* cell densities for experiments.

2.10 Cell culture for mammalian cells:

GM 12878 human B Lymphocyte cells were cultured in liquid DMEM medium with 10 v/v % FBS and incubated at 37°C in 5% CO₂ atmosphere surrounding. Cells with different densities were added into 96-well plates for oxygen respiration test.

2.11 Cell respiration measurements:

Micelles dialyzed in 10 mM HEPES buffers were added to the cell culture media to make the AIE fluorophore concentration of 6.6 μM and PTTTFPP concentration of 0.83 μM, corresponding to the polymer concentration of 0.096 mg/mL. Cell numbers or densities were adjusted to tune the oxygen consumption time. For avoiding the oxygen exchange between those dissolved in medium and air, mineral oil was used to seal the plate. For comparison, cell respiration without oil seal was also measured. 405 nm was used as an excitation wavelength, 520 nm was collected for AIE fluorophores' emission and 650 nm was collected for PTTTFPP's emission using either bottom or top reading. Experiments were carried out at 37°C. LB medium was used for *E. Coli* respiration and DMEM medium was used for GM 12878 human B Lymphocyte cells' respiration. We found the cell respiration velocity were not affected by the top reading or bottom reading, however, bottom reading results in more significant sensor

photobleaching than top reading at high cell density when cell density is higher than 1×10^7 cfu/mL. So most of our experiments, top reading is used except specifically pointed out.

3. Results and discussion:

3.1 Polymer synthesis

We prepared a graft polymer (**P3**) for incorporation of hydrophobic PtTFPP into micelles to enable their applications in aqueous solutions (**Scheme 1**). PtTFPP is one of the highest efficient oxygen probes, however hydrophobic, limiting its direct applications for biostudies. Using the approach of particles and micelles is one of the ways to enable its applications in water. Herein we extended to new graft polymers (instead of block copolymers) with a capability of easily manipulating as a new approach for macromolecular oxygen probe development. We prepared the **P3**, which contains green-emitting AIE fluorophores. The AIE fluorophores act as internal references for the oxygen responsive probes of PtTFPP in the micelles to achieve ratiometric sensors. In this materials' system, we also extended the applications of the AIE fluorophores into oxygen sensing research fields. Following Scheme 1, **P3** was synthesized. The polymers were characterized using ^1H NMR, and GPC for their structures and molecular weights. PCL segments were chosen here as represent hydrophobic and biocompatible blocks, and PHPMA chains were chosen as typical biocompatible and hydrophilic polymers. PCL and PHPMA were well used for drug delivery and bioimaging, we extended those for sensing research.

3.2 Micelle preparation and characterization

Using a dialysis approach as described in the experimental part, PtTFPP was

successfully incorporated into the micelles (**Figure 1A**) with the hydrophilic PHPMA as the shells and the hydrophobic PCL with/without AIE fluorophores as the cores. Critical micelle concentration (CMC) of the polymer **P3** was measured to found to be 5.0 $\mu\text{g/mL}$ (supporting information Figure S1), comparable with other block copolymers and graft copolymers [53-55]. The release of the PtTFPP probes for the micelles were studied at 37°C. It was found less than 1.5% of PtTFPP was released from the micelles for the first 10 hours and about 2% of PtTFPP was released from the micelles after 20 hours (supporting information Figure S2), or, in other words, more than 98% of PtTFPP was retained in micelles, showing high stability of the micelles for application. Sizes of the micelles were found to 34 to 52 nm, increasing with the increase of PtTFPP concentrations (**Figures 1B and 1C**).

3.3 Photophysical properties

AIE properties were investigated as shown in **Figure 2**. The polymer in DMSO had weak emission with a low quantum yield of 0.02. At the same polymer concentration, with the increase of water fraction in DMSO, fluorescence intensity increases (Figure 2A). These phenomena were observed in other AIE materials [38, 52] suggesting the typical AIE characteristics. The quantum yield of **P3** in DMSO/H₂O (2: 98 by volume) increased to 0.13, which is 6.5 times greater than that in pure DMSO. Figure 2B showed typical fluorescence intensities' changes with the changes of DMSO/H₂O ratios. All the polymer segments, PHPMA, PCL, and PAIE have good solubility in DMSO. The AIE fluorophores can rotate freely in DMSO to lose their energies resulting in their weak emission intensities. With the addition of water into the polymer solutions in DMSO, the hydrophobic AIE fluorophores and

PCL started to self-assemble with the hydrophilic PHPMA polymer chains through the formation of micellar nanostructures. The hydrophobic PAIE segments aggregate together in the PCL domain as the hydrophobic cores, limiting or restricting the intramolecular rotations of the AIE fluorophores to increase the fluorescence intensities. With water fractions reached to 40%, the aggregations almost became compact. With further increase of water fractions, fluorescence intensities did not change further. This observation indicated typical characteristics of the AIE phenomenon and revealed that the AIE phenomenon was caused by the restricted intramolecular motions of the luminogens in their aggregate states, which blocked the nonradiative decay channels and thus enhanced fluorescence intensities.

The fluorescence spectrum of the AIE chromophore overlaps with both the Soret and Q-band absorptions of **PtTFPP** (**Figure 3**), indicating that efficient FRET can occur from the AIE chromophore as the FRET donor to PtTFPP as the FRET acceptor at a suitable condition. However, just simply mixing the two fluorophores in their individual micelles did not result in any FRET (supporting information Figure S3). This is due to that FRET will occur from donor (herein AIE fluorophore) to acceptor (PtTFPP) only when the distance between the donor to acceptor is less than 10 nm [58].

PtTFPP is insoluble and does not fluoresce well in an aqueous solution. However, once it was encapsulated within a micelle, the aqueous solution began to fluoresce. For this study, the concentration of **AIE** was kept constant. When the concentration of **PtTFPP** in **P3** (simplified as **PtTFPP/P3**) was increased, the fluorescence intensity of the AIE chromophore decreases (**Figure 3B**). The energy transfer efficiency can be calculated to be as high as 97%. This indicates that effective FRET indeed occurs within these micelles. It also proves that the

nanoconfinement provided by the AIE copolymer helps the efficient generation of PtTFPP's excited state through energy transfer. Furthermore, the dual fluorophores in the micelles also provides ratiometric sensing for oxygen. We found a phenomenon of that with the increase of PtTFPP concentration in **P3** polymer, the emission peaks of AIE fluorophore shifted to short wavelength significantly. Most likely, this might be aggregation styles of the AIE fluorophore is manipulated by PtTFPP, resulting J-aggregations [59] of the AIE fluorophores by interacting with PtTFPP. It is quite interesting to find that quantum yields of PtTFPP in the micelles were 0.20 and 0.02 under nitrogen and air, respectively. It was reported that quantum efficiency of PtTFPP in CH₂Cl₂ under nitrogen was 0.088 [56], while a sugar modified PtTFPP, which is soluble in water, was reported to have a very low quantum yield of 0.001 [60]. Quantum efficiency is affected by a few factors such as solvents, solvent polarity, fluorophore-fluorophore interactions, ordered and disordered aggregations [61]. Normally, the aggregated compounds show weaker emissions or exhibit lower fluorescence quantum yields than the monomeric fluorophore, due to a nonradiative decay. However, in the presence of micelles, the aggregates could be disrupted to their monomeric forms because of the distributions of fluorophores by the micellar cores formed from the hydrophobic polymers. This process will increase fluorescence quantum efficiency. When PtTFPP molecules were incorporated into the micelles, the PLC micellar cores forced the fluorophores to aggregate in the hydrophobic regions. However in the meantime, the PCL polymers could also have disrupted their aggregations. On the other hand, the polarity of PCL could also have affected the quantum yield. As a result of the combination effects of these, the quantum yield of PtTFPP in the micelles was higher than those in CH₂Cl₂. This result showed that the micelles

provided an excellent environment to enable the application of hydrophobic PtTFPP in aqueous solution and maintain its high efficiency.

3.4 Oxygen responses of PtTFPP in micelles

Dissolved oxygen concentrations were adjusted using a mixture gas of oxygen and nitrogen. The response to oxygen was shown in Figure 4A. We chose a system of PtTFPP/P3 (0.1/4.8 by weight corresponding to PtTFPP/AIE by molar ratio of 1/4.4) as a typical example. In this system, AIE fluorophore emission is weak, so there is no influence of the emission of AIE on PtTFPP during the titration. Clearly, the emission of PtTFPP decreased with the increase of oxygen concentration, while the emission of AIE fluorophore did not change. The intensity ratio (I_0/I) curves (Figure 4B) follows the Stern-Volmer equation as given in equation 2. The linear response to oxygen makes an easy calculation for an unknown oxygen concentration measurement.

$$\frac{I_0}{I} = 1 + K_{SV}[O_2] \quad (2)$$

where K_{SV} is the Stern-Volmer quenching constant. I_0 and I are the steady-state luminescence signals measured in the absence of oxygen and presence of different oxygen concentrations, respectively. $[O_2]$ is the dissolved oxygen concentration tuned by the nitrogen and oxygen mixed gas. Based on the emission intensity changes of the PtTFPP probe only and the ratiometric intensity changes by AIE/PtTFPP, K_{SV} s were calculated to be 0.412 and 0.023 L mg^{-1} , respectively. According to their standard derivations (δ), the limits of detections (LODs) for DO were calculated to be 0.056 and 0.054 mg/L, respectively by using $3\delta/K_{SV}$.

3.5 Reversibility and responsive time test

Using the air/nitrogen saturation method (for comparison with the approach with glucose oxidase, we called this method as dynamic reversibility test), it was found the response times, t_{90} (i.e., the time for 90% of the total change in fluorescence intensity to occur) and t_{95} (i.e., the time for 95% of the total change in fluorescence intensity to occur), were 30 and 33 s from the deoxygenated condition to the air saturated condition (Figure 5A), respectively. Recovery times from the air saturated solution to deoxygenated solution were slower with a t_{90-r} of 100 s and t_{95-r} of 132 s. These response times were quite faster than those of PtTFPP physically doped in poly(2-hydroxyethyl methacrylate) (PHEMA) matrix and faster than those of PtTFPP physically doped in polystyrene (PS) matrix [62]. The t_{95} of PtTFPP in PHEMA and PS matrices from deoxygenated solutions to oxygenated solutions are 50 and 84 seconds, respectively [62]. This comparison demonstrated that these micelles are reasonable materials for oxygen sensing. Through the reversibility test, it was found the oxygen probe of PtTFPP is stable, while the AIE fluorophore is relatively unstable compared with that of PtTFPP. There is about 5% of the intensity decrease of the AIE probe during the 1 hour experimental period, most likely due to photobleaching and/or oxidation during the continuous exposure to oxygen atmosphere. Thus for real application, it might be better if long time continuous exposure should be alleviated.

Stationary state oxygen responsive time was studied using the deoxygenated approach with glucose oxidase through the oxidation of glucose and consumption of oxygen (Figure 5B) without oil sealing or stirring. Depending on the concentration of glucose oxidase, fast response time from air to deoxygenated condition was observed as fast as 0.7 s, showing the fast responses of the sensors. This response time of 0.7 s is faster than that the fast response

time of 4 s when PtTFPP was incorporated in polystyrene-*block*-polyvinylpyrrolidone beads suspended in water although experimental condition might be different [28]. A conversion of the fluorescence intensities in Figure 5B with glucose oxidase concentration of 0.1 mg/mL to absolute oxygen concentrations was given in supporting information Figure S4.

3.6 Cell respiration:

A strain of ampicillin-sensitive *E. coli* JM109 was used for cell respiration study. Sensor solution was directly added into cell culture medium and then fluorescence signals (intensities) were read using a plate reader under 405 nm excitation. AIE emission was detected at 520 nm and PtTFPP emission was measured at 650 nm. The measurement was carried out at 37°C. Previously, we have showed that similar PHPMA copolymers were cell impermeable for a few cell lines, like *E. coli* and HeLa cell lines [63]. For avoiding the exchange of oxygen in the cell culture medium with air, a thin layer of mineral oil was added on the top of the LB medium. The use of mineral oil to prevent gas diffusion for biostudies was reported in literatures [33,48]. For comparison, a series of experiments were carried out without using the oil. When there is no oil sealing, the oxygen consumption was much slower (about 2/3) than those with oil sealing (supporting information Figure S5), thus for the following discussion, the experiments were performed with oil sealing.

Figure 6A shows one typical experiment to demonstrate the micelles are capable for oxygen respiration measurement. It was found fluorescence intensities of the AIE probes and PtTFPP probes decreased slightly during the first 20 minutes. This decrease is caused by the temperature equilibrium (increase) to 37°C. This is quite common for many organic

fluorophores, of which their intensities decreased slightly with an increase of temperature. After temperature equilibrates, emissions of the two probes become stable. The AIE fluorophore's emission did not change with/without bacteria, showing it is a good internal reference for oxygen sensing. The PtTFPP emission did not change at the condition without bacteria, while intensity increased with the experimental time when bacteria were in medium. The increase of the PtTFPP intensity is caused the oxygen consumption by bacteria. After the oxygen is consumed completely, the oxygen probe PtTFPP's intensity decreased slightly at high cell density while kept flat at low cell density with time showing the minimum oxygen exchange with air and slight photo-degradation under our experimental condition (Figure 6B). The photo-instability of the sensors at continuous photo-irradiation is relevant to cell density: the higher cell density, the significant decay. Thus, for long time application, using relative low cell density is preferred.

With the demonstration of the capacity of the sensor for cell respiration measurement, we used the oxygen sensor to measure oxygen respiration under different cell densities (Figure 6B). It was observed clearly that oxygen was consumed faster at higher cell densities. For the initial *E. coli* cell density of 5×10^7 cfu/mL, the dissolved oxygen was completely consumed within 15 min. According to the rate of oxygen depletion by *E. coli* and initial cell densities, oxygen consumption rate of single cell was calculated to be from 4.72×10^{-18} to 6.44×10^{-18} mol cfu⁻¹ s⁻¹ with oil sealing and 3.14×10^{-18} to 4.32×10^{-18} mol cfu⁻¹ s⁻¹ without oil sealing, slightly affected by the initial cell densities. It is known oxygen consumption rate depends on particular bacteria and experimental conditions including temperature and time. Geckil et al. [64] reported that the oxygen consumption rate of *E. coli* was in a range of

$1.21 \times 10^{-19} - 1.78 \times 10^{-18} \text{ mol cfu}^{-1} \text{ s}^{-1}$, showing the influences of experimental conditions and cell viabilities/species at different labs.

We further demonstrated whether this sensor can be used for detection of *E. coli* in water in real time, which is important to detection of food contamination. As shown in Figure 6C, it was found the sensor can detect at least 100 cfu/mL *E. coli* in medium during 7 hours, the higher cell density the sooner detection. The cell density and detection time shows linear relationship (Figure 6D), making it easy to predict time at an estimated contamination level. The capability of bacteria detection at a concentration of 100 cfu/mL meets the requirement the National Standard of the People's Republic of China (GB 5749-2006) for the Standards for Drinking Water Quality. It requires the total bacterial count should be smaller than 100 cfu/mL in drinking water after culturing at 37°C for 24 hours.

The sensor is not only suitable for bacteria but also suitable for mammalian cells. We used GM 12878 human B Lymphocyte cells as a typical cell line. Such cells are suspension cells, easily manipulating cell densities for our test. Results showed the higher cell density consumed faster for environment oxygen as given in Figure 7.

3.7 Cell toxicity

We tested cell viability with a constant cell density (5×10^7 cfu/mL) while different sensor concentrations ranging from 5 – 40 $\mu\text{g/mL}$ of PtTFPP, corresponding to PtTFPP concentration of 5 to 40 μM (Figure 8A) and with a constant sensor concentration of 40 $\mu\text{g/mL}$ at different cell densities ranging from 3×10^6 to 1×10^8 cfu/mL (Figure 8B). No clear cell inhibition or cytotoxicity was observed to the cells at our experimental conditions. It

should be noted here, that the common concentration of the PtTFPP in **P3** used for our studies are between 1 to 5 $\mu\text{g/mL}$. The cytotoxicity of the micelles to GM 12878 human B Lymphocyte cells were also tested using MTT assay, no obvious cytotoxicity was observed (supporting information Figure S6)

3.8 Potential drugs/antibiotics screening using the sensor through oxygen consumption measurements

Antimycin is a potent electron transport chain (ETC) inhibitor and an antibiotic to various bacteria cells [65, 66]. It was also known that antimycin A inhibits the growth of various cells via stimulating oxidative stress-mediated death [65, 66]. Herein, we tested the inhibition of an antibiotic, antimycin, on the oxygen respiration using *E. coli* as a representative cell line. It is expected the sensor can be used for drug scanning through the observation of oxygen consumption rates' differences. For this study, we mixed antimycin with various concentrations with *E. coli* (initiator cell density of 10^6 cfu/mL) and monitored the oxygen consumption (Figure 9). It was found that without the adding of antimycin, cells consumed oxygen completely around 3 hours; while with the increase of antimycin concentrations, the oxygen consumption was slowed down significantly. When antimycin's concentration reached 100 $\mu\text{g/mL}$ in the cell culture medium, oxygen consumption was completely inhibited. Thus this study indicated the sensor has potential application for antibiotics, toxins, and drug scanning through the oxygen consumption measurements.

4. Conclusion

A graft polymer with PCL as the hydrophobic polymer segment, PHPMA as the hydrophilic polymer chain, and an AIE fluorophore as the internal reference probe was synthesized. This polymer was used to incorporate a high efficient however hydrophobic oxygen probe of PtTFPP to enable the application of the oxygen probe in aqueous solution to achieve ratiometric oxygen sensing and maintain high brightness of the PtTFPP in aqueous solution. The ratiometric sensor was used to measure oxygen consumption of *E coli* and GM 12878 human B Lymphocyte cells, concentration dependent oxygen consumption was clearly observed. The sensor is also capable to *in-situ* detect bacteria to at least 100 cfu/mL within 7 hours and to screen drugs/antibiotics. There are no obvious cytotoxicity observed, moreover the probe of PtTFPP in the micelles exhibited high quantum efficiency up to 20% under nitrogen, which is the ever reported highest quantum efficiency for PtTFPP probe. Since the sensor is in liquid format, it is easily used in many commercially available common instruments like plate readers for hypoxia, oxygen respiration, and oxygen consumption measurement at high-throughput for further biology understating, disease diagnosis, and metabolism investigation.

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Scheme 1. Synthesis of the graft copolymer **P3** for nanosensor preparation.

Figure 1. Preparation of PtTFPP encapsulated micelles (A); DLS of the micelles with a constant **P3** concentration of 4.8 mg/mL while variable PtTFPP concentrations (B); and a plot of micelles sizes against PtTFPP concentrations derived from B (C).

Figure 2. AIE properties of **P3**. (A) The emission intensity change of **P3** in the mixture of DMSO and water. Emission spectra were excited at 405 nm. (B) Plot of emission peaks at 525 nm against water percentage.

Figure 3. A) The normalized UV-Vis and steady fluorescence spectra of the AIE fluorophore as the donor in **P3** and PtTFPP as acceptor encapsulated by **P3** in aqueous media; B) The fluorescence spectra of PtTFPP in **P3** with various loading amounts of PtTFPP ranging from 0 to 0.5 mg/mL in 5 mg/mL of **P3** under the illumination of 405 nm in water; C) FRET efficiency against the loading amounts of PtTFPP in **P3** in aqueous solutions.

Figure 4. Oxygen responses of the micelles excited at 405 nm from de-oxygenated condition (labeled using N_2 corresponding to 0 mg/mL DO) and oxygenated condition (labeled using O_2 corresponding to 40.95 mg/mL DO) (A) and Stern-Volmer plots by using the oxygen probe's emission intensities at 650 nm (I_0/I at 650 nm) and by using the AIE fluorophores' intensities at 520 nm to PtTFPP's emissions at 650 nm for ratiometric plots (I_{520}/I_{650}) (B). I_0 is the intensity at 650 nm from the PtTFPP probe under de-oxygenated condition; I is the intensity at 650 nm under a certain dissolved oxygen (DO) concentration. I_{520} is the intensity at 520 nm from the AIE fluorophore under a certain DO concentration and I_{650} is the intensity at 650 nm from the PtTFPP probe under a certain DO concentration. The FRET system was composed by **P3**/PtTFPP (4.8/0.1 by weight). Concentrations of oxygen used for this titration were 0, 4.1, 8.2, 12.3, 16.4, 20.5, 24.6, 28.7, 32.8, 36.9, and 41.0 mg/L respectively.

Figure 5. Reversibility and response time tests of the PtTFPP/**P3** under an excitation at 405 nm through the purging of nitrogen and oxygen (A) and response times using glucose (0.25 M) and glucose oxidase with various concentrations (B).

Figure 6. A) Representative fluorescence intensities' changes at 650 nm (PtTFPP channels) and at 525 nm (AIE channels) with and without *E. coli*; B) *E. coli* concentration dependent oxygen consumption velocities at PtTFPP channels only; C) oxygen consumption used for low concentrated *E. coli* detection; D) plots of *E. coli* concentration dependent detection time.

Figure 7. GM 12878 Human B Lymphocyte cells' oxygen consumption in 200 μ L DMEM medium measured at 37°C.

Figure 8. A) Cell growth of *E. coli* with the same initial OD with different PtTFPP/P3 concentrations, 40.5 $\mu\text{g/mL}$ represent PtTFPP concentration in the micelles corresponding to a polymer **P3** concentration of 2 mg/mL; B) Cell growth of *E. coli* at different initial OD with the same PtTFPP/P3 concentration (5 $\mu\text{g/mL}$ of PtTFPP and 0.24 mg/mL of **P3**, the normal concentration we used for studies).

Figure 9. Inhibition of oxygen consumption test using antimycin as a typical antibiotic at different concentrations. Cell initial concentration is of 1×10^6 cfu.