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Highly efficient ratiometric extracellular oxygen sensors through physical incorporation of a conjugated polymer and PtTFPP in graft copolymers

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ABSTRACT

An amphiphilic graft copolymer (**P3**) composed of poly(oligo (ethylene glycol) methacrylate) (POEGMA) as a hydrophilic segment and a polystyrene (PS) as a hydrophobic chain was prepared. **P3** was used to incorporate a highly efficient hydrophobic oxygen probe platinum(II)-5,10,15,20-tetrakis-(2,3,4,5,6-pentafluorophenyl)-porphyrin (PtTFPP) in its micelles to enable the application of PtTFPP in aqueous solution for biosensing. For achieving ratiometric oxygen sensing, a hydrophobic conjugated polymer (**CP**) was used as a fluorescence resonance energy transfer (FRET) donor for PtTFPP - the FRET acceptor. Results showed that diameters of these nano-oxygen sensors ranging from 50 to 60 nm and FRET efficiency as high as 98% could be achieved. Further, FRET contributed to the brightness of PtTFPP in micelles. High quantum yields of PtTFPP in non-FRET micelles and FRET micelles under nitrogen were achieved to be 0.109 and 0.231, respectively. The sensors were found to be able to measure oxygen concentrations from 0.082 to 40.9 mg/L at room temperature with linear Stern-Volmer constants. These sensors were also demonstrated to be suitable for monitoring extracellular oxygen consumption of *E. Coli* and HeLa cells. This study illustrated a feasible approach to achieve highly efficient ratiometric oxygen sensors without specific structure modification of the efficient PtTFPP probe, however using an amphiphilic graft copolymer approach.

1. Introduction

Oxygen is one of most critical element for numerous fields such as environment, engineering, ocean, industry, agriculture, biology and health. In biological systems, oxygen is a crucial metabolic substrate in living aerobic cells and tissues. With the improvement of scientific research, the influence of oxygen concentration for cells can be learned gradually. For instance, although oxygen is essential to metabolism and respiration, it is also known that elevating oxygen concentration is toxic for a variety of cell types [1]. Most of aerobic and microaerophilic organisms would develop their protective mechanism to survive at hyperoxia environment. When oxygen concentration surpasses the air saturation level, superoxide (O_2^-) and hydrogen peroxide (H_2O_2), called reactive oxygen species (ROS), accumulate as byproducts which are toxic to organisms because they are more active than molecular oxygen [2–4]. Oppositely, hypoxia, which is a reduction of oxygen level in cells or tissues, leads to physiological and pathological consequences ranging from impairment of aerobic and microaerophilic functions to hypoxia ischemic tissues injury and even cell death [5]. Hypoxia has been found to associate with multifarious diseases, such as acute and chronic vascular diseases, neurological disorders, pulmonary diseases and cancer [6,7]. Thus, the ability to measure oxygen level in living cells and tissues precisely is crucial for understanding cellular activity, accessing etiology of types of diseases, and diagnosing cancer. It is well known that cellular respiration is a suit of metabolic reactions and processes taking place in the cells of organisms or tissues to convert bioenergy from nutrients into adenosine triphosphate (ATP). Therefore, measuring cellular oxygen consumption is important to assess aerobic glycolysis rates, oxidative phosphorylation, and also for high-throughput drug screening [8–10].

For sensing, quantum yield is one concern. The hydrophilic oxygen probes made of metal porphyrins (PtCPK, PtCP, PdCPK, PtTCPP)

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[11,12] showed extremely low quantum yields as low as in the range of 0.001 to 0.0095. For achieving high quantum yields and endow some new features for oxygen sensors, Vinogradov et al. presented a few series of oxygen sensors with dendrimer shapes [13–15]. 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. Peripheral PEGylation or carboxylication of the dendrimers ensures high aqueous solubility and prevents interactions of the probes with biological macromolecules. As a result, quantum yields of the porphyrin-containing dendrimers are around 0.017 to 0.073 [14]. At 2012, we used nanostructured micelles of an amorphous block copolymer of poly(ε-caprolactone)-*block*-poly (ethylene glycol)to encapsulate a highly efficient however extremely hydrophobic oxygen probe of platinum(II)-5,10,15,20-tetrakis-(2,3,4,5,6-pentafluorophenyl)-porphyrin (PtTFPP) to enable the application of PtTFPP in aqueous solution with high efficiency of form 0.107 to 0.111 [16]. Very recently, we used a graft copolymer of poly(ε-caprolactone) as a hydrophobic chain and poly(*N*-(2-hydroxypropyl)methacrylamide) as a hydrophilic chain to encapsulate the hydrophobic PtTFPP to achieve quantum yield of 0.20 under nitrogen [17]. As compared with amphiphilic block copolymers widely used for nanostructures formation, graft copolymers have the similar capability to form nanostructures. A few merits of graft copolymers at least include that (1) graft copolymers can be easily prepared and (2) their structured can be widely manipulated. Some amphiphilic graft copolymers were used for incorporation of quantum dots for cell imaging and drug delivery [18–20].

On the other hand, fluorescence resonance energy transfer (FRET) approach was found to be efficient for achieving ratiometric measurements with high accuracy and brightness [21–24]. In literature, some conjugated polymers/metal porphyrin pairs were reported and investigated either by single photon [25–27] and two-photon excitation approaches [28,29]. Especially, Ruslan et al reported the use of conjugated polymer nanoparticles to achieve ratiometric oxygen sensing and high quantum yield of 18% [26]. Almost all of these nanostructured oxygen sensors were reported for intracellular sensing and imaging.

Herein, we prepared new FRET-based highly efficient, nanostructured, and ratiometric oxygen sensors with a focus on extracelluar oxygen sensing. We developed a new graft copolymer by using polystyrene as hydrophobic segments and poly(oligo(ethylene glycol)) as hydrophilic chains. The polymer contain a small portion of poly(methacrylic acid)s for potential modification for further applications in the future (Scheme 1). This polymer was used to incorporate a hydrophobic conjugated polymer (CP) as FRET donor and the widely-used oxygen probe of PtTFPP as the electron acceptor to achieve highly efficient FRET-based nanostructured oxygen probe (Fig. 1) with high quantum yield. For demonstrating the bioapplication capability of the sensor, the sensor was applied to monitor cell respiration through a commercially available plate-reader.

2. Experiment

2.1. Materials and reagents

PtTFPP was purchased from Frontier Scientific (Logan Utah). 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was ordered from Sigma-Aldrich (St. Louis, MO). Dialysis membranes (regenerate cellulose, Mw cut off 10,000) were purchased from Pierce (Rockford, IL). All the chemicals and solvents in this work were of analytical grade. Methacryloyl chloride, 2-hydroxyethyl methacrylate (HEMA), azobisisobutyronitrile (AIBN), methacrylic acid, tetrakis(triphenylphosphine) palladium, 4,7-dibromobenzo[*c*]-1,2,5-thiadiazole triethyl amine, 9,9-dihexylfluorene-2,7-diboronic acid bis(1,3-propanediol) ester, N,N,N′,N″,N″-Pentamethyldiethylenetriamine (PMDETA), 2-hydroxyethyl 2-bromo-2-methylpropanoate, and anisole were commercially available from Aldrich and used without further purification. CuBr was also purchased form Aldrich and purified by washing with glacial acetic acid followed by 2-propanol, and then dried under vacuum. Oligo(ethylene glycol) methyl ether methacrylate (OEGMA, M_n = 1000 g/mol) was passed through a column of activated basic alumina to remove inhibitors. Doubly distilled water was used for 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer and Lysogeny Broth (LB) medium preparation. Exact gas percentage was controlled using a gas manipulator (measuring error: ± 1%) purchased from Alicat Scientific (Tucson, Arizona, USA). All measurements of sensing behaviors were performed at room temperature (23 ± 1 °C) under an atmospheric pressure (760 mm Hg, or 101 kPa).

LB medium made of 10 g tryptone, 5 g yeast extract and 10 g NaCl in 1 L distilled water was used for *E. coli* cultures. Dulbecco's Modified Eagle Medium (DMEM) which was purchased from Invitrogen (Carlsbad, CA) was used for HeLa cell culture. HEPES buffer solution was made of 2.383 g HEPES in 1 L distilled water. The pH value of buffer solution was adjusted to pH 7.4 using sodium hydroxide for monitoring oxygen consumption of *E.coli* and living cells bioimaging.

2.2. Instruments

400 M ^{1}H NMR (Ascend™ 400, Bruke) was used to confirm the structure of amphiphilic graft polymer and its precursors. UV/Vis spectrometer (Lambda 25, PerkinElmer) and spectrofluorophotometer (FluoroMax-4, Horiba) were used to measure the absorbance and fluorescence intensity, respectively. Dynamic light scattering (DLS) (Nano ZS, Malvern) and gel permeation chromatography (GPC) (Water 1515, Waters) were used for measurements for micelle diameters and polymer molecular weights. The solution was passed through a 0.45 μm Nylon microfilter (VWR, Batavia, IL) to remove dust before the DLS measurements. Cell imaging multi-mode microplate reader (Cytation 3, Cytation™) was used for monitoring oxygen consumption of *Escherichia coli* (*E. coli*) bacteria and HeLa cells. Confocal fluorescence microscope (TCS-SP8, Leica) was used for fluorescence imaging of living cells.

2.3. Synthesis

2.3.1. Synthesis of hydroxyl-containing polymer P1

It was prepared according to published procedures [30]. Styrene (1.14 mL, 0.010 mol), CuBr (57.2 mg, 0.004 mol) and 1 mL anisole were added to a 10 mL of Schlenk flask, equipped with a stir bar. After sealing it with a rubber septum, the flask was frozen with liquid nitrogen. Subsequently, the flask was degassed and backfilled with nitrogen. 2-Hydroxyethyl 2-bromo-2-methylpropanoate (35 μL, 0.002 mol) as an initiator and PMDETA (83 μL, 0.004 mol) as a complexing agent were injected into the flask, respectively. The mixture was then frozen with liquid nitrogen and degassed by three freeze-pump-thaw cycles, and the vial was placed in an oil bath and stirred at 90 °C for 10 h under nitrogen. The reaction was stopped by exposing to air after dilution with CH_2Cl_2 and the solution was filtered through a column filled with alumina. The polymer was recovered by precipitation in methanol and dried in a vacuum oven at 50 °C for 12 h. Yield: 740 mg (71%). $M_{n\ (GPC)}$ = 3792, $M_{n\ (NMR)}$ = 3200. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.89 (dd, J = 252.4, 49.6 Hz, 1 H), 4.46 (s, 1 H), 3.76 (s, 1 H), 2.60 – 1.12 (m, 1 H).

2.3.2. Synthesis of methacrylate-containing polymer P2

300 mg of **P1** and 1.12 mL of triethylamine were dissolved in 5 mL methylene chloride. 0.7 mL of Methacryloyl chloride was added into the mixture solution dropwise in ice bath. Macromonomer **P2** was precipitated into methanol from the CH_2Cl_2 solution after 10 h reaction at room temperature. Yield: 240 mg (80%). $M_{n\ (GPC)}$ = 3792, $M_{n\ (NMR)}$ = 3200. ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.89 (dd, J = 252.4, 49.6 Hz, 1 H), 6.10 (s, 1 H), 5.57 (s, 1 H), 4.46 (s, 1 H), 4.10 (s, 1 H),