Invited paper

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Optical oxygen sensing with quantum dot conjugates

<https://doi.org/10.1515/pac-2018-0303>

Abstract: The ability to track and quantify changes in oxygen concentration as a function of disease progression or therapy is crucial to advance targeted chemotherapeutics. New non-invasive sensors must be developed that are small enough to penetrate into tissue and monitor dynamic changes with high resolution in real time. One way to address this challenge is with the use of nanoparticle-based sensors. This review details the design, synthesis, and characterization of optical oxygen sensors that combine a fluorescent semiconductor quantum dot (QD) with an oxygen-responsive phosphorescent molecule. The QD may have multifaceted roles in these constructs, serving as an internal standard for ratiometric sensing, as an antenna for multiphoton absorption, and as an energy transfer donor for the attendant phosphorescent molecule. Solid-state devices may be prepared by embedding the two components in a polymer matrix. Alternatively, solution-phase sensors can be synthesized by covalent conjugation, self-assembly in organic solvents, or micelle encapsulation in aqueous media. Several sensors have been used for biological imaging and oxygen sensing, demonstrating that these constructs can quantify oxygen in biological systems.

Keywords: FRET; IUPAC-SOLVAY International Award for Young Chemists; oxygen sensor; porphyrin; quantum dot; self-assembly; two-photon spectroscopy.

Introduction

The concentration of oxygen is a crucial parameter in biological systems. In cells of aerobic organisms, oxygen serves as the terminal electron acceptor for the process of mitochondrial oxidative phosphorylation, which generates ATP as an energy storage molecule to drive other metabolic processes. In this series of reactions, stepwise electron-transfer events shuttle electrons from NADH and FADH₂ to O₂, resulting in its four-electron, four-proton reduction to water [1]. However, uncoupling of the pathway can generate partially reduced oxygen species, such as superoxide and peroxide. These are commonly referred to as reactive oxygen species (ROS) and are sources of oxidative stress. Fortunately, nature has evolved enzymes, including superoxide dismutase and catalase, as well as cytosolic small molecules (e.g. glutathione and ascorbate) to scavenge these ROS [2, 3]. Thus, it is critical for cells to maintain oxygen levels at an appropriate concentration to maximize the productive use of electrons for ATP synthesis while limiting the generation of toxic ROS.

The partial pressure of oxygen (pO₂) from inspired air is approximately 160 Torr, which then decreases to 104–150 Torr in the lungs [4]. Tissues are oxygenated by arterial circulation (50–100 Torr) and the venous system (20–40 Torr) returns blood to the lungs for reoxygenation [5]. Most tissues exhibit pO₂ in the 5–20 Torr range [4], utilizing elaborate metabolic and physiological processes to maintain oxygen homeostasis.

Article note: A collection of peer-reviewed articles by the winners of the 2017 IUPAC-SOLVAY International Award for Young Chemists.

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If cells encounter abnormally low oxygen tensions or hypoxia ($pO_2 \leq 5$ Torr), they utilize a series of adaptive strategies that include arresting the cell cycle, secreting survival and proangiogenic factors, and reducing energy consumption [6]. Hypoxia inducible factors (HIFs) are a family of proteins that regulate gene transcription associated with the response to hypoxic stress [7, 8]. While hypoxia represents a stress to many cell types, some cells natively inhabit hypoxic environments. One such example is the stem cell niche, which is defined as the dynamic environment that regulates signaling cues and enables stem cells to persist, grow, and change their fate based on the organism's physiological needs [9]. Hypoxia has been shown to activate molecular pathways that regulate stemness (e.g. Oct4 and Notch) in several types of stem cells [10]. Cultures of embryonic stem cells grown under hypoxic conditions maintain pluripotency, while those grown in normoxic conditions exhibit differentiation [11]. These observations suggest that stem cell proliferation and quiescence may be regulated by oxygen gradients within the niche. Low oxygen levels may also cause stromal cells in the niche to secrete factors that support stem cell pluripotency [12]. Thus, hypoxia serves as a protective feature that enables stem cells to persist in an undifferentiated state and to minimize mutations and oxidative damage.

Conversely, chronic hypoxia can be a consequence of cancer pathophysiology, where the heterogeneous microenvironment of solid tumors exhibits low pO_2 . Tumor vasculature is heterogeneous, dilated, and leaky, leading to inefficient delivery of blood and oxygen that results in heterogeneous blood flow and characteristically low oxygenation in tumors [13]. Hypoxia is a master regulator of tumor progression through the stimulation of angiogenesis and metastasis, maintenance of stem-like cell phenotypes, reduction in tumor cell apoptosis, and suppression of the native immune response [13–15]. Consequently, hypoxia is an attractive target for cancer chemotherapy because it plays a significant role in both tumor development and therapeutic resistance [16, 17]. The concentration of oxygen, glucose, and pH represent three key parameters that define the metabolic status of a tumor and serve as direct measures of respiration, nutrient consumption, and metabolism, respectively [18]. While most normal cells rely on mitochondrial oxidative phosphorylation to meet their energy demands, tumor cells exploit aerobic glycolysis. This phenomenon was first observed by Otto Warburg nearly a century ago and is now commonly known as the Warburg effect [19, 20]. As a consequence, the tumor microenvironment is characterized by low extracellular pH (6.6–6.8), arising from the accumulation of both lactic acid and carbonic acid because the abnormal tumor vessels cannot efficiently remove these metabolites [21]. Together, tumor acidosis and hypoxia incapacitate immune cells, promote metastatic cell phenotypes, and induce the expression of angiogenic factors that trigger and stimulate tumor growth [22–24].

Metabolic profiles of tumors can provide spatiotemporal maps of key markers for tumor growth and their response to therapy. Thus, there is an imperative to develop novel sensors that are small enough to penetrate into solid tumors and accurately quantify analytes of interest [25]. To this end, fluorescent semiconductor quantum dots (QDs) are ideal scaffolds for the development of nanosensors because of their favorable optical properties: high photoluminescence quantum yields and photostability, broad excitation profiles, and narrow emission bands [26]. In the past several years, many research groups have published general reviews on biosensing and bioimaging with QDs, including Nocera [27, 28], Mattoussi [29–31], Credi [32, 33], Medintz [34], von Borczyskowski [35], Snee [36], and Chen [37]. The scope of this review is limited to QD-based oxygen sensors and their biological applications. For general oxygen sensing references, the interested reader is directed to recent reviews by Quaranta [38], Papkovsky [39], Wolfbeis [40], and Evans [41].

Sensor design criteria

In order to accurately measure oxygen levels in biological systems, several design elements must be incorporated. An ideal optical sensor should utilize red or near-infrared (NIR) light in the 600–1100 nm range, as tissue is transparent to these wavelengths [42]. This enables deep tissue imaging because photon absorption and scatter is minimized, resulting in maximal signal from the sensor. Ideally, both the excitation and emission wavelengths of the sensor would fall in the tissue transparency window. Two-photon microscopy satisfies this requirement, as it utilizes NIR excitation light. Moreover, this technique provides high spatial

resolution ($\sim 1\ \mu\text{m}$) to accurately map the heterogeneity of biological microenvironments [43–45]. However, most common organic fluorophores are poor absorbers under two-photon excitation [46, 47]. Semiconductor quantum dots (QDs), such as CdSe or CdTe, have two-photon absorption cross-sections that are many orders of magnitude higher than those of traditional fluorophores [48]. As a result, QDs are attractive photon antennas for multiphoton microscopy. Moreover, they have additional unique optical properties, resulting in their widespread use for bioimaging [49, 50].

Perhaps the most important design element for a sensor is the ability to detect the analyte of interest over a desired concentration range. Since QDs are typically insensitive to changes in their environment [51], they must be paired with an analyte-responsive dye to render a functional sensor. This dye is selected on the basis of its optical properties (e.g. emission in the tissue transparency window) and analyte sensitivity over the desired concentration range. For the sensor to function, there must be communication between the QD and the attendant dye. Förster resonance energy transfer (FRET) [52] is a common method of signal transduction in QD/fluorophore dyads [27]; the energy absorbed by one component is non-radiatively transferred to the other without emission of a photon. With two fluorophores in a single construct, the emission intensity ratio of the two components may serve as a quantitative measure of analyte concentration, furnishing a ratiometric sensor. This is a robust sensing methodology that is independent of sensor concentration and is functional in scattering media, such as cells and tissue [28]. The following subsections will review these design principles, which are then applied to the QD-based oxygen sensors described herein.

Semiconductor quantum dots

Inorganic semiconductor nanocrystals, commonly referred to as quantum dots (QDs), are photostable fluorophores with high photoluminescence quantum yields. QDs have broad excitation profiles with high molar absorption coefficients (10^5 – $10^6\ \text{M}^{-1}\ \text{cm}^{-1}$) and very narrow ($\sim 30\ \text{nm}$ FWHM), Gaussian-shaped emission profiles (Fig. 1a) [26]. This is in stark contrast to traditional molecular fluorophores, which exhibit narrow absorption profiles with broad emission features that tail to the red and are prone to photobleaching. The emission profile of QDs is tunable with particle size as a result of quantum confinement (Fig. 1b). When a bulk semiconductor absorbs a photon with energy greater than the band gap, an electron is promoted from the valence band (VB) to the conduction band (CB), resulting in the formation of a free hole and free electron. The electron is then attracted to the localized positive charge by Coulombic interactions, forming an exciton or bound electron–hole pair. As a result of this stabilizing interaction, the exciton has a lower energy than the free electron and free hole [53]. The spatial extension of the exciton is defined by the Bohr radius. For

Fig. 1: (a) Absorption (solid lines) and emission spectra (dotted lines) of a CdSe QD in toluene (—) and Nile blue, a molecular fluorophore, in ethanol (—). The QD exhibits a broad absorption spectrum and sharp emission profile. Conversely, the molecular fluorophore exhibits a broad emission spectrum that tails to the red region of the spectrum. (b) Schematic representation of quantum confinement. As the size of the particle decreases, the exciton becomes confined and behaves like a particle in a box ($E \sim 1/r^2$). The effective band gap E_g increases and discrete, molecule-like energy levels form at the band edges. Reprinted with permission from [28]. Copyright 2014 American Chemical Society.

example, a photogenerated exciton in CdSe will delocalize over a distance of about 12 nm. When the size of the semiconductor particle is on the order of the Bohr radius, the wave function of the exciton is perturbed, modulating the optical properties of the material [54, 55]. The confinement energy of the exciton in the particle becomes larger than the Coulomb energy, giving rise to molecule-like states rather than bands for the bulk material. As a result, the exciton behaves like a particle in a box; the energy (E) depends on the size (r) of the box ($E \sim 1/r^2$) and the effective band gap (E_g) increases with decreasing particle size [56]. Additionally, discrete energy levels arise at the band edges, resembling a molecular HOMO–LUMO gap [26, 55]. In the case of CdSe, small QDs (~2 nm diameter) emit blue light, while large QDs (~6 nm) emit red light [55]. The range of accessible emission wavelengths depends on the band gap of the semiconductor. For example, UV emission is possible with high band gap materials like CdZnS [57], while IR emission can be achieved with low band gap materials like PbS or PbSe [58]. For a more detailed discussion of QD photophysics, the reader is directed to a recent review by Klimov and coworkers [59].

One of the most common ways to synthesize QDs is by the rapid injection of organometallic precursors into a solvent such as tri-*n*-octylphosphine oxide (TOPO) in conjunction with a stabilizer (a phosphonic acid or hexadecylamine) at elevated temperatures (>300 °C) [26, 60]. Alternatively, octadecene (ODE) may be used as the solvent with oleic acid or oleylamine as a stabilizer. This results in a short burst of homogenous nucleation of nanocrystallites. The sudden drop in temperature from adding room-temperature reagents to the hot reaction mixture prevents further nucleation, maintaining a narrow size distribution of the nanoparticles. Continued heating of the reaction mixture results in nanocrystal growth that may proceed by focused growth and/or Ostwald ripening. Under focused growth conditions, the temperature of the system is low enough to preclude the nucleation of additional particles and there are sufficient remaining precursors. As a result, smaller particles grow at a faster rate than larger particles. Conversely, the precursor concentration is low during Ostwald ripening. The high surface energy of the small particles promotes their dissolution, providing the necessary material to redeposit on larger particles [61]. Since the average size and distribution of particles depends on the growth conditions, the optical properties of the QD can be tuned with fidelity. QDs are often overcoated with another semiconductor in a core–shell motif to further enhance the optical properties by passivating surface defects. If a QD core is coated with a semiconductor that has a higher band gap, then a photogenerated exciton will be confined to the core, preventing it from interacting with the environment and thereby increasing the photoluminescence quantum yield [55, 60]. For example, CdSe QDs overcoated with CdS or CdZnS exhibit stable emission with near-unity (>95 %) quantum yields [62, 63]. Conformal shells are usually synthesized using layer-by-layer methods, such as successive ion layer adsorption and reaction (SILAR), which enable the preparation of precise overcoats [64].

As synthesized, quantum dots are hydrophobic and are natively capped with the solvent used in their synthesis. Consequently, the QD surface must be modified to impart water solubility and biocompatibility. This may be achieved by adding a shell of silica in a core–shell motif [65] or using mercaptoacetic acid as a surface ligand [66]. The native hydrophobic capping ligand may be exchanged with a multidentate hydrophilic ligand [67–70]. Such ligands may be polymeric and incorporate several functional groups for QD surface binding, water solubility, and further derivatization. One example is a dihydrolipoic acid–polyethylene glycol (DHLLA–PEG) polymer [71, 72]. While DHLLA is commonly used to solubilize QDs in aqueous media, such QDs are not readily derivatized, are unstable at pH < 6, and exhibit nonspecific binding in cell cultures. The inclusion of a PEG unit stabilizes the QD in solutions of pH 5.0–9.5 and preserves high photoluminescence quantum yields. Moreover, the PEG polymer may be terminated with an additional functional group to enable conjugation of an analyte-responsive dye [72]. Alternatively, water solubility may also be achieved by encapsulating hydrophobic QDs with oligomeric phosphines [73], dendrimers [74–76], phospholipids [77], or amphiphilic polymers [78, 79].

Oxygen-responsive phosphorescent molecules

Several methodologies have been used to quantify oxygen in biological systems: electron paramagnetic resonance (EPR), positron emission tomography (PET), magnetic resonance imaging (MRI), hemoglobin

saturation spectrometry, polarographic microelectrodes, and phosphorescence quenching [41]. Of these methods, phosphorescence quenching is non-invasive and offers the highest spatiotemporal resolution. When a photon is absorbed (A), a molecule is promoted to an excited singlet state (S_1) (Fig. 2a). It can then undergo vibrational relaxation (VR) to thermally release excess energy and subsequently fluoresce (F) from the lowest vibrational level of S_1 . Alternatively, intersystem crossing (ISC) can occur to access the triplet manifold (T_1). Then, as with fluorescence, the molecule can undergo VR to relax to the lowest vibrational state and subsequently phosphoresce (P). In a competing process, the triplet can interact with molecular oxygen (a ground state triplet, 3O_2) via collisional quenching in a triplet–triplet annihilation process, generating singlet oxygen (1O_2) and returning the molecule back to the ground state (S_0). Consequently, the triplet state will emit fewer photons at higher concentrations of oxygen. This phosphorescence quenching serves as a quantitative measure of pO_2 and this process follows Stern–Volmer kinetics [80]:

$$\frac{\tau_0}{\tau} = 1 + k_q \tau_0 [O_2], \quad (1)$$

where τ_0 is the natural radiative lifetime of the phosphorescent molecule in the absence of O_2 , τ is the phosphorescence lifetime at a given oxygen concentration $[O_2]$, and k_q is the bimolecular quenching rate constant.

In order to have an efficient optical oxygen sensor, the molecule should undergo rapid intersystem crossing upon excitation to maximize the population of the triplet state. The inclusion of heavy atoms will facilitate ISC by the so-called “heavy atom effect” through increased spin–orbit coupling, which scales with atomic number (Z^n). As a result, the population of the triplet manifold increases at the expense of the singlet, resulting in fluorescence quenching. However, the molecule must phosphoresce rather than simply non-radiatively relax back to the ground state without emitting a photon. This can be achieved with closed-shell

Fig. 2: (a) Jablonski diagram illustrating photophysical processes that can occur after photon absorption. Photonic processes are drawn with straight arrows: absorption (A), fluorescence (F), and phosphorescence (P). Non-radiative processes are denoted with wavy arrows: vibrational relaxation (VR) and intersystem crossing (ISC). Chemical structures of common oxygen-responsive phosphorescent molecules: (b) homoleptic bipyridine (bpy) complexes, (c) heteroleptic phenanthroline (phen)/bipyridine (bpy) complexes, (d) tetraphenylporphyrin, (e) mesoporphyrin IX, (f) triphenylcorrole, and (g) pyrene.

coordination complexes of second and third row transition metals. Figure 2 shows the chemical structures of common oxygen-responsive compounds. Perhaps the most widely utilized phosphorescent molecules are Ru(II) and Os(II) polypyridine complexes (Fig. 2b and c), which possess triplet metal-to-ligand charge transfer ($^3\text{MLCT}$) states with lifetimes up to $6.4\ \mu\text{s}$ and quantum yields as high as 30 % in the case of $[\text{Ru}(\text{dpp})_3]^{2+}$, where $\text{dpp} = 4,7\text{-diphenyl-1,10-phenanthroline}$ [81]. In general, Os(II) derivatives emit at longer wavelengths than the analogous Ru(II) complexes, but they have significantly shorter lifetimes ($\sim 10^2\ \text{ns}$) and, consequently, lower quantum yields ($\phi \sim 1\%$) [38]. With lifetimes of several microseconds, Ru(II) polypyridine complexes are sensitive over the 0–760 Torr O_2 range [82].

In addition to polypyridine complexes, metalloporphyrins (Fig. 2d and e) are common phosphorescent molecules for oxygen sensing applications. While a variety of metals have been utilized, including Ir(III) [83] and Gd(III) [84], the most prevalent examples are Pd(II) and Pt(II) because of their strong room-temperature phosphorescence in the 650–800 nm range and long ($\sim 10^2\ \mu\text{s}$) phosphorescence lifetime [85]. In general, longer phosphorescence lifetimes increase the sensitivity of the sensor at lower oxygen concentrations. These attributes render Pd(II) and Pt(II) porphyrins responsive over the biologically relevant 0–160 Torr O_2 range and emissive in the tissue transparency window. In general, Pd(II) porphyrins have longer lifetimes, redder emission profiles, and higher oxygen sensitivity than their Pt(II) analogs. Conversely, Pt(II) porphyrins have higher quantum yields than their Pd(II) congeners [40]. Commercial Pd(II) porphyrins (Oxyphor R2) and benzoporphyrins (Oxyphor G2), which are available as glutamate dendrimers, are water-soluble sensors that have been used for solution-based sensing applications [86]. Another approach to make these porphyrins water soluble is to incorporate them into a protein scaffold. Mesoporphyrin IX complexes (Fig. 2e) of Ru(II) [87], Pd(II) [88], and Pt(II) [88] have been incorporated into the heme binding pocket of myoglobin and horseradish peroxidase to furnish oxygen-responsive proteins. Recently, corroles (Fig. 2f) have also been explored as oxygen sensors. Corroles are 18 π -electron tetrapyrrole macrocycles, like porphyrins, but contain a contracted 23-atom core. As a result, corrole is a trianionic ligand when fully deprotonated, in contrast to the dianionic porphyrin ligand. Consequently, Au(III) corroles, which are isoelectronic with Pt(II) porphyrins, have been reported as optical oxygen sensors [89]. These complexes emit in the 780–795 nm region with triplet lifetimes of $\sim 80\ \mu\text{s}$, rendering these effective sensors over the 0–160 Torr O_2 range. Similarly, Os(VI) $\equiv\text{N}$ corroles are oxygen sensors that emit in the same spectral range with longer lifetimes and higher quantum yields [90].

One additional class of oxygen-responsive compounds is polycyclic aromatic hydrocarbons (PAH), such as perylene and pyrene (Fig. 2g) [38, 40]. These molecules typically emit in the near-UV to blue region of the spectrum and have high fluorescence quantum yields. For example, pyrene exhibits an emission maximum at 393 nm with $\phi = 65\%$ [91]. The emission profiles of PAHs may be red shifted by increasing the size of the aromatic π system (e.g. $\lambda_{\text{max,em}} \sim 445\ \text{nm}$ for perylene [92]). While most typical fluorophores have excited state lifetimes between 1 and 10 ns that are independent of oxygen, PAHs have $\tau > 100\ \text{ns}$ that are readily quenched by oxygen. This is due to a triplet–triplet annihilation process known as P-type delayed fluorescence [93]. The “P” designation stands for pyrene [94], the first molecule that was discovered to exhibit this phenomenon [91]. In this process, two molecules in the triplet state interact to form an excited state complex that subsequently dissociates to give one molecule in the excited state and one molecule in the ground state. As a result, fluorescence is observed on the timescale of triplet states, enabling optical oxygen sensing. It should also be noted that molecular oxygen induces ISC in certain PAHs (e.g. 9,10-diphenylanthracene), resulting in fluorescence quenching [95, 96].

Förster resonance energy transfer

If an oxygen-responsive phosphorescent molecule is coupled to a QD, then energy must be transferred between the two components to observe oxygen-dependent emission from QD excitation. One of the most common methodologies for signal transduction is Förster resonance energy transfer (FRET) [52]. In this mechanism, energy is non-radiatively transferred via long-range dipole–dipole interactions from a donor (D) fluorophore to an acceptor (A) molecule (Fig. 3a), resulting in a decrease of the donor’s emission intensity.

Fig. 3: (a) Schematic of a QD donor (D)/fluorophore acceptor (A) construct. The QD serves as a photon antenna and transfers the absorbed energy to the acceptor, promoting it to an excited electronic state. (b) Illustration of spectral overlap between donor emission (—) and acceptor absorption (—). (c) Molecular orbital representation of FRET. Adapted with permission from [28]. Copyright 2014 American Chemical Society.

Upon absorption of a photon, one of the electrons in the HOMO of the donor is promoted to the LUMO (Fig. 3c). During FRET, the donor returns to the ground state without emitting a photon, while, simultaneously, the acceptor is promoted to an excited state [97]. In a FRET-based sensor, the donor is selected for its absorption properties, while the acceptor is chosen based on its emission properties and analyte sensitivity [27, 28]. The FRET pair is selected to maximize the spectral overlap between donor emission and acceptor absorption (Fig. 3b) for efficient energy transfer.

Mathematically, the FRET efficiency (E) may be quantified in terms of energy transfer rates or donor–acceptor distances:

$$E = \frac{mk_{D-A}}{mk_{D-A} + \tau_D^{-1}} = \frac{mR_0^6}{mR_0^6 + r^6}, \quad (2)$$

where m is the number of acceptor molecules per donor molecule, k_{D-A} is the rate of energy transfer, τ_D is the lifetime of the donor in the absence of acceptor, R_0 is the Förster distance (or the distance at which FRET efficiency is 50%), and r is the distance between the donor and acceptor [97]. Based on this equation, E increases with increasing values of m , which can be readily achieved in QD-based sensors by incorporating many acceptor molecules per QD donor. When $m > 1$, the parameter r reflects the ensemble average of D–A distances. The FRET efficiency may be determined experimentally by measuring the lifetime of the donor:

$$E = 1 - \frac{\tau_{D-A}}{\tau_D}, \quad (3)$$

where τ_{D-A} is the lifetime of the donor in the presence of acceptor. The Förster distance R_0 can be calculated for a given donor–acceptor pair:

$$R_0^6 = \frac{9000(\ln 10)\kappa^2\Phi_D}{128\pi^5 N n^4} \int_0^\infty F_D(\lambda) \varepsilon_A(\lambda) \lambda^4 d\lambda, \quad (4)$$

where κ^2 is the relative orientation factor of the transition dipoles (taken to be 2/3 for random D–A orientations), Φ_D is the fluorescence quantum yield of the donor, N is Avogadro's number, n is the refractive index of the solvent, $F_D(\lambda)$ is the normalized emission intensity of the donor, and $\varepsilon_A(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength λ . The latter half of this equation is known as the spectral overlap integral and is often denoted by the variable J . Critical FRET length scales for R_0 typically range from 2 to 9 nm [97]. Since the emission profile of QDs is tunable with size, QDs are attractive FRET donors because the overlap integral may be tuned with fidelity.

In an alternative to the dipolar Förster interaction, energy may be transferred in an exchange-driven process known as Dexter energy transfer [98]. This mechanism relies on wave function overlap and requires close contact between the donor and acceptor. This can be achieved by using high concentrations of both donor and acceptor, or through direct physical or chemical contact between the two components. In canonical

Dexter energy transfer, an excited electron in the LUMO of the donor is transferred to the acceptor molecule. The acceptor then transfers an electron from its HOMO to the HOMO of the donor, resulting in an excited-state acceptor and ground-state donor. Typically, Dexter energy transfer is associated with fluorescence quenching and can be observed if J is small, as electron transfer rates then become significant. When J is high, FRET will occur before Dexter transfer [97]. This has been demonstrated experimentally with a series of QDs of varying size conjugated with a surface-bound squaraine dye [99]. QD photoluminescence experiments demonstrated that smaller QDs (low spectral overlap and high orbital overlap with the dye) exhibit higher energy transfer efficiencies than predicted by FRET and faster rates of energy transfer. These results suggest that Dexter transfer dominates in these constructs. Conversely, in conjugates with larger QDs (high spectral overlap and low orbital overlap with the dye), FRET is the predominate mechanism of energy transfer. This example demonstrates that both mechanisms may be operative in QD conjugates, but the dominant mode of energy transfer depends on spectral and orbital overlap.

Two-photon spectroscopy

The theory of two-photon absorption was first proposed by Maria Göppert-Mayer in 1931 [100]. It was not until 30 years later that this concept was experimentally verified when Kaiser and Garrett used red light from a ruby laser to excite a sample of Eu(II)-doped CaF_2 and detected blue fluorescence [101]. Until 1990, multiphoton spectroscopy was considered an exotic technique that was limited to the fields of optical spectroscopy and chemical physics. This was because the availability of photon sources that could provide sufficiently high peak powers to increase the probability of multiphoton absorption was limited [97]. With the advent of mode-locked solid-state femtosecond lasers (e.g. Ti:sapphire), the accessibility of multiphoton spectroscopy has dramatically increased [46]. In 1990, two-photon imaging of biological samples was first reported using an ultrafast laser source [102]. Since then, multiphoton laser scanning microscopy (MPLSM) has become a powerful, routine imaging technique that uses NIR light to exploit the tissue transparency window and enable deep tissue imaging (450–600 μm) with approximately 1 μm spatial resolution [42, 44, 45, 103]. Given the small focal volume of two-photon excitation (*vide infra*), photobleaching and photodamage are minimized while generating high-resolution images to accurately map the heterogeneity of the tumor microenvironment [104–106].

Under linear or one-photon excitation, a molecule is directly promoted to the first excited state through the absorption of a photon with an energy comparable to the HOMO–LUMO gap (Fig. 4a). Multiphoton processes require two or more photons to simultaneously interact with a molecule. In a two-photon process, the

Fig. 4: Comparison of one- versus two-photon excitation processes. (a) A fluorophore is promoted to an excited state by a single blue photon and subsequently emits a green photon. (b) The same transition in (a) is achieved with two red photons whose total energy is equivalent to that of the blue photon. (c) One- and (d) two-photon excitation of fluorescein. Under linear excitation, fluorescence is observed throughout the sample. Conversely, two-photon excitation exhibits sharp, pinpoint emission at the focal point of the excitation source, highlighted with a yellow circle. Reprinted with permission from [28]. Copyright 2014 American Chemical Society.

first photon excites the molecule to an intermediate state while the second photon promotes the molecule to its final state (Fig. 4b). The intermediate state can be an eigenstate of the molecule (i.e. a real state for sequential two-photon absorption) or a superposition of molecular states (i.e. a virtual state for simultaneous two-photon absorption) [46]. The intensity of a two-photon transition, or the two-photon absorption cross-section σ_2 , is analogous to the molar absorption coefficient ε for linear excitation and can be estimated using a single intermediate state approximation [107]:

$$\sigma_2 \approx \sigma_{ij}\sigma_{jf}\tau_j, \quad (5)$$

where σ_{ij} is the one-photon absorption cross-section for the transition from initial state i to intermediate state j , σ_{jf} is the one-photon absorption cross-section for the transition from intermediate state j to final state f , and τ_j is the lifetime of the intermediate state and determines the time scale for photon coincidence. For a virtual state, $\tau_j \leq 10^{-15}$ s and simultaneous photon absorption occurs. Alternatively, sequential photon absorption can occur if the intermediate state is a real state with a lifetime of 10^{-9} to 10^{-12} s. A one-photon absorption cross-section σ_1 may be estimated using the length of the transition dipole, which is $\sim 10^{-8}$ cm for a typical organic fluorophore, to give $\sigma_1 \sim 10^{-16}$ or 10^{-17} cm². Using these values and Eq. 5, the corresponding two-photon absorption cross-section may be estimated as 10^{-49} cm⁴ s/photon or 10 Göppert-Mayer (GM), where 1 GM = 10^{-50} cm⁴ s/photon.

Under linear excitation, the excitation density in the focal region is proportional to the intensity of the incident light. Since the excitation density depends on the square of the intensity for a two-photon process, the excitation density rapidly decreases as the distance from the focal point increases. As a result, the two-photon excitation volume is significantly smaller than that of one-photon excitation, thereby increasing the spatial resolution of the signal. This phenomenon is illustrated in Fig. 4c and d. Under one photon excitation, a streak of emission is visible along the focal plane, while emission is visible only at the focal point of the excitation source under two-photon excitation. Consequently, two-photon excitation minimizes photobleaching and photodamage.

While multiphoton imaging is a powerful technique, there are few dyes that have sufficiently high two-photon absorption cross-sections. Most typical fluorophores have $\sigma_2 \sim 10$ –100 GM [45, 47, 108, 109]. For fluorescein, a common dye used in biological labeling, σ_2 varies from 8 GM to 37 GM over the 690–960 nm range [108]. Some conventional fluorophores exhibit unusually high cross-sections, such as Cy3 and Rhodamine 6G, which have maximal σ_2 values of 140 GM and 150 GM, respectively, under 700 nm excitation [108]. Although organic molecules have been developed with $\sigma_2 \sim 10^3$ – 10^4 GM [110, 111], common analyte-responsive dyes have prohibitively low two-photon absorption cross-sections for MPLSM applications. Conversely, QDs possess large two-photon absorption cross-sections [112–114], with σ_2 as high as 47 000 GM for CdSe/ZnS core-shell QDs [48]. This property renders QDs suitable two-photon antennas for an appended analyte-sensitive dye to enable biological sensing with MPLSM.

Solid-state sensors

The most common types of optical oxygen sensors are solid-state devices where the phosphorescent molecule is immobilized in a polymer matrix, on a solid surface, or in mesoporous silica [85]. Therefore, it is not surprising that the majority of QD-based oxygen sensors are solid-state constructs where a QD and O₂-responsive phosphorescent molecule are blended in a polymer or sol-gel matrix. Since there are two emissive components, the construct is ratiometric; the QD serves as an internal emission intensity standard and the ratio of QD to phosphorescent molecule emission is a quantitative measure of pO₂. This is a significant improvement over sensors with only a phosphorescent molecule that rely on changes in absolute emission intensity. The first sensor of this type consisted of a CdSe/ZnS QD ($\lambda_{em} = 520$ nm) and [Ru(bpy)₃]Cl₂ blended in a sol-gel matrix and coated on a glass slide [115, 116]. A later iteration of this sensor design utilized [Ru(dpp)₃]Cl₂ as the phosphorescent molecule with CdTe QDs ($\lambda_{em} = 680$ nm) [116].

Solid-state sensors have primarily utilized Pd(II) and Pt(II) porphyrins because they offer greater oxygen sensitivity than Ru(II) polypyridine complexes. Both metalloporphyrins have been paired with CdSe QDs

Fig. 5: Optical output of a solid-state sensor strip comprised of a Pt(II) porphyrin and a CdTe/CdS QD. The top row shows the emission of the Pt(II) porphyrin alone, while the bottom row shows the ratiometric output of the combined porphyrin/QD sensor. Adapted with permission from [118]. Copyright 2008 Wiley-VCH Verlag GmbH & Co. KGaA.

($\lambda_{\text{em}} = 515 \text{ nm}$) in an organically modified silica (ormosil) xerogel and coated on a fiber optic probe [117]. A ratiometric sensor strip was prepared by coating a glass slide with CdTe/CdS QDs ($\lambda_{\text{em}} = 552 \text{ nm}$) dispersed in a poly-silane. This optical reference layer was then coated with an ormosil matrix containing a Pt(II) porphyrin ($\lambda_{\text{em}} = 648 \text{ nm}$). The sensor exhibited a gradual red to green optical transition with increasing pO_2 (Fig. 5) [118]. Variations on these methods have also led to the development of dual sensors for measuring oxygen in conjunction with another analyte of interest. For O_2 and temperature sensing, CdSe QDs ($\lambda_{\text{em}} = 532 \text{ nm}$) overcoated with a silica shell and a Pt(II) porphyrin were embedded in a sol-gel matrix. The QD exhibited temperature-dependent emission over the 30–100 °C range. At constant temperature, QD emission serves as an internal intensity standard for ratiometric oxygen sensing [119]. A similar approach was utilized for measuring oxygen and Cu(II) ions, where the photoluminescence of CdSe QDs ($\lambda_{\text{em}} = 580 \text{ nm}$) was quenched by copper ions [120]. A later iteration of this sensor included a coumarin dye as an internal reference that was invariant to both analytes [121]. Dual O_2 and H_2O_2 detection was accomplished by blending CdSe/ZnS QDs ($\lambda_{\text{em}} = 517 \text{ nm}$) and $[\text{Ru}(\text{dpp})_3]\text{Cl}_2$ in an ethyl cellulose matrix and coating on an optical fiber. QD photoluminescence was quenched by H_2O_2 and served as the means of detection of this analyte [122].

In addition to these fiber optic and other monolithic devices, ratiometric oxygen sensors have been prepared in polymeric micro- or nanoparticles. The first example of this type of sensor was reported by McShane and co-workers in 2011 [123]. Amine-functionalized porous silica microspheres (10.3 μm diameter) were treated with QDs ($\lambda_{\text{em}} = 786 \text{ nm}$) and a Pt(II) porphyrin, both bearing carboxylic acid moieties for conjugation via amide bond formation. One advantage of this sensor is that both components emit in the NIR. The sensor exhibited a linear optical response at $[\text{O}_2] < 400 \mu\text{M}$ (240 Torr). Recently, a ratiometric nano-sensor was reported where a cyclometalated Ir(III) complex was used as the O_2 -responsive phosphorescent molecule [124]. QDs ($\lambda_{\text{em}} = 690 \text{ nm}$) and $\text{Ir}(\text{piq})_3$ ($\text{piq} = 1\text{-phenylisoquinoline}$ with $\lambda_{\text{em}} = 620 \text{ nm}$) were dispersed in a liquid crystalline phase of monooleoylglycerol; sonication was employed to exfoliate particles with a 35.6 nm average diameter. The small size of the particles makes the sensor amenable for biological imaging applications (*vide infra*). The 750 nm to 620 nm emission intensity ratio was used to quantify pO_2 and this parameter exhibited a linear response from 1 to 21 % (8–160 Torr) O_2 .

In all of the sensors discussed so far, the QD serves as an internal intensity standard for ratiometric sensing, as the phosphorescent molecule and QD are spectrally separated and do not interact. There has been one example of a solid-state sensor where the QD was utilized as a FRET donor. A CdSe/CdS QD ($\lambda_{\text{em}} = 590 \text{ nm}$) was selected such that its emission profile overlapped with the absorption of Pt(II) octaethylporphyrin ketone and the two components were blended in a 1:3 ratio in a polyvinyl chloride (PVC) matrix [125, 126]. This sensor has been utilized for simultaneous O_2 sensing and voltage monitoring in biological tissue (*vide infra*). Since this sensor operates via FRET, it is functional under both one- ($\lambda_{\text{ex}} = 480 \text{ nm}$) and two-photon ($\lambda_{\text{ex}} = 850 \text{ nm}$) excitation. The 590 nm to 757 nm emission ratio served as a quantitative measure of pO_2 [126].

Covalent conjugates

While solid-state sensors may be the most prevalent, these devices are generally not amenable for biological sensing, particularly for *in vivo* applications. Instead, a soluble, solution-based sensor is preferred so that it may be efficiently distributed throughout a biological sample. One of the most common ways in which to

functional groups including imidazole, amines, and carboxylic acids on its surface. An acceptor molecule with one of these functional groups can exchange with the hydrophobic ligands on the QD surface. The first example of this type of sensor was reported by Credi and co-workers in 2011 [130]. A hydrophobic, TOPO-capped CdSe/ZnS QD ($\lambda_{\text{em}} = 636 \text{ nm}$) was treated with imidazole-functionalized pyrene ($\lambda_{\text{em}} = 398 \text{ nm}$), which binds directly to the QD surface. As with the PAH sensors described the previous section, the two components are spectrally separated and do not interact via FRET. The sensor is functional over the 0–760 Torr O_2 range and the 632 nm to 398 nm emission ratio serves as a suitable parameter for ratiometric sensing.

In order to red shift the emission profile of the sensor and increase the sensitivity at physiological O_2 pressures, Nocera and co-workers paired Pd(II) porphyrins ($\lambda_{\text{em}} = 690 \text{ nm}$) with CdSe core-shell QDs ($\lambda_{\text{em}} = 519 \text{ nm}$) [131]. Pyridyl groups at the *meso* positions of the porphyrin enable direct binding to the QD surface (Fig. 7). The sensors were prepared by adding 10 equivalents of porphyrin per QD. It was found that porphyrins with two pyridyl substituents in a *cis* arrangement have high binding affinity ($K_A \sim 10^7 \text{ M}^{-1}$), whereas porphyrins with a single pyridyl group exhibit weaker binding. Time-resolved porphyrin emission demonstrates that there are two populations in the self-assembled constructs, corresponding to surface-bound (75 %) and free porphyrin (25 %). This is also reflected in titration data, which exhibits binding saturation with ~ 8 equivalents of added porphyrin. The QD was selected such that its emission profile overlaps with Pd(II) porphyrin absorption ($\lambda_{\text{abs}} = 523 \text{ nm}$), resulting in high FRET efficiencies (94 % with $R_0 = 4.1 \text{ nm}$). As a result, the sensor functions under two-photon excitation ($\lambda_{\text{ex}} = 800 \text{ nm}$), while emission from the porphyrin alone is undetectable. Under ambient air, porphyrin phosphorescence is significantly quenched and the emission intensity increases 100-fold in the absence of oxygen, rendering the sensor sensitive over the 0–160 Torr O_2 range. Since QD photoluminescence is unaffected by O_2 , ratiometric sensing is possible [131].

This approach was then extended to Au(III) corroles to further shift the optical readout of the sensor to the NIR ($\lambda_{\text{em}} = 780 \text{ nm}$) [132]. Since Au(III) corroles have redder absorption profiles than those of Pd(II) porphyrins [89], a yellow-emitting CdSe/ZnS QD was selected ($\lambda_{\text{em}} = 572 \text{ nm}$) to maximize spectral overlap and FRET efficiency. Corroles with a *meso*-carboxylic acid group enable high-affinity binding to the QD surface with $K_A \sim 10^6 \text{ M}^{-1}$. Conversely, the corresponding methyl ester exhibits drastically diminished affinity ($K_A \sim 10^4 \text{ M}^{-1}$), as this molecule can only associate with the QD through non-specific hydrophobic interactions. This difference in affinity is also reflected in the FRET efficiencies and average donor-acceptor distance (r): 84 % ($R_0 = 5.12 \text{ nm}$, $r = 5.70 \text{ nm}$) for the carboxylic acid and 22 % ($R_0 = 5.03 \text{ nm}$, $r = 9.12 \text{ nm}$) for the corresponding methyl ester. The high FRET efficiency renders the sensor functional under two-photon excitation ($\lambda_{\text{ex}} = 965 \text{ nm}$) [132]. Since these QDs are larger than those used for the Pd(II) porphyrin sensors [131], binding saturation is not observed, even up to 20 equivalents of corrole. Additionally, monoexponential decay kinetics are observed for Au(III) corrole emission in these constructs, indicating that all the corrole molecules are bound to the QD. The oxygen sensitivity of these conjugates is comparable to that of the free Au(III) corrole and is responsive over the 0–160 Torr O_2 range [132].

Fig. 7: Schematic representation of the QD–Pd(II) porphyrin self-assembled sensor. Upon porphyrin binding, QD photoluminescence is quenched and the absorbed energy is transferred to the surface-bound porphyrin by FRET, promoting it to an excited state. Porphyrin phosphorescence is quenched by oxygen while the QD is unaffected, resulting in a ratiometric optical response. Reprinted with permission from [131]. Copyright 2013 American Chemical Society.

Micelle-encapsulated sensors

The sensors described in the previous section rely on the hydrophobicity of the QD for self-assembly and are functional in organic solvents rather than in aqueous media. However, another self-assembly process may be leveraged to solubilize those sensors in water: micelle formation. Micelles are comprised of amphiphilic molecules that assemble such that the hydrophobic ends of the molecule reside in the micelle interior, while the hydrophilic groups on the exterior interact with solvent. Through hydrophobic interactions with surface ligands, natively capped QDs can readily be solubilized in water by micelle encapsulation. Phospholipids have been used to accomplish this and these micelles are amenable for biological imaging [77]. This method has been used to encapsulate the self-assembled sensors with Pd(II) porphyrins [133] and Au(III) corroles [132] described in the previous section. The organic-soluble sensors were treated with a PEG-2000-functionalized phosphoethanolamine lipid using sonication processing to afford sensors with a hydrodynamic diameter of 18.2 nm in the case of Pd(II) porphyrins (Fig. 8). Electron microscopy demonstrates that a single QD is contained within each micelle. Based on the optical spectra of the sensors, there are eight porphyrins per QD [133], which is consistent with the photophysical characterization of the organic-soluble sensors [131]. The sensor exhibits 99 % FRET efficiency ($R_0 = 4.06$ nm) to give an average donor–acceptor distance of 2.67 nm. This is identical to the QD to porphyrin center-to-center distance based on structural metrics, where the QD radius is 1.90 nm and the distance between the pyridyl nitrogen and the Pd(II) center is 0.77 nm. Under linear excitation, two porphyrin populations are observed: one that is surface-bound to the QD and one that is dispersed in the phospholipids of the micelle. Conversely, a single population of porphyrins is observed under two-photon excitation ($\lambda_{\text{ex}} = 850$ nm), as only the QD-bound porphyrins are sufficiently close to the QD to be excited via FRET. As a result, two-photon excitation is ideal for *in vivo* applications of this sensor, as the experiment naturally selects for a single population of porphyrins that is directly associated with the QD, simplifying the photophysics of the system. An oxygen-dependent ratiometric response is achieved with the 682 nm to 528 nm ratio. The sensor was calibrated at both 25 °C and 37 °C to give k_q values of $9.02 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ and $1.36 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$, respectively. This latter value was used to quantitatively measure oxygen concentrations *in vivo* (*vide infra*).

Similarly, the Au(III) corrole self-assembled sensors were encapsulated in the same phospholipid. It was found that both the carboxylic acid and methyl ester derivatives were incorporated into the micelle constructs,

Fig. 8: Schematic representation of the micelle-encapsulated QD–Pd(II) porphyrin sensor. Oxygen can freely diffuse into the micelle and quench the Pd(II) porphyrin. QD photoluminescence is unaffected, resulting in a ratiometric response. Reprinted with permission from [133]. Copyright 2015 American Chemical Society.

demonstrating that direct surface binding is not a prerequisite for micelle encapsulation. However, the carboxylic acid, which directly binds to the QD surface, resulted in higher FRET efficiency and closer average donor–acceptor distances (r) than the methyl ester: 93 % ($R_0 = 4.13$ nm, $r = 3.94$ nm) and 66 % ($R_0 = 4.06$ nm, $r = 5.24$ nm), respectively [132]. The D–A distance of 3.94 nm determined from the FRET analysis is consistent with the structural metrics of the construct, where the QD radius (2.88 nm) and the distance between the carboxylic acid group and the Au(III) center (1.11 nm) results in a center-to-center distance of 3.99 nm [134]. As in the case of the Pd(II) porphyrin micelles [133], two corrole populations are observed under linear excitation and correspond to QD-bound corrole and free corrole dispersed in the lipids of the micelle. The surface-bound corrole population serves as a reliable metric to measure pO_2 and this population exhibits a k_q value of $1.6 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ [132].

While micelle-encapsulation of self-assembled sensors is a facile way to produce water-soluble QD conjugates, there are several limitations to this methodology. First, the acceptor must be appropriately functionalized to enable sufficient QD surface binding and to maximize incorporation in the micelle. Moreover, the size of the QD limits the number of acceptors that can be accommodated on the surface. For example, only ~8 Pd(II) porphyrins could bind to the surface of a 3.5 nm diameter QD [133], while at least 20 Au(III) corroles can bind to the surface of a 5.8 nm diameter QD [132]. If the quantum yield of the acceptor is low, the emission may not be comparable with QD photoluminescence, resulting in QD emission that is off-scale relative to the phosphorescent molecule. In the case of the Au(III) corrole sensors, QD emission ($\phi = 0.70$) is substantially greater than corrole phosphorescence ($\phi = 0.0024$) and 20 equivalents are insufficient to quench QD photoluminescence and increase corrole emission such that the intensity of the two components are comparable [132]. Additional acceptor molecules are needed than can be physically accommodated on the QD surface. Finally, micelle constructs can only be used to sense analytes that readily diffuse through the lipid layer to reach the analyte-responsive dye buried in the micelle core. To overcome these limitations, the micelle approach can be extended beyond the encapsulation of self-assembled constructs by utilizing phospholipids modified with functional groups at the end of the PEG chain. In this way, the QD can readily be solubilized in water and covalent conjugation strategies may be used to functionalize the exterior of the micelle with acceptor molecules. By using a mixture of functionalized and unfunctionalized lipids, the number of surface groups may be adjusted to easily tune the donor–acceptor ratio. This method has been applied to the preparation of a sensor construct where a water-soluble Au(III) corrole with carboxylic acid moieties was covalently attached to a micelle-encapsulated QD with surface amines. As a result, more corroles were coupled to the QD than are possible via self-assembly methods, resulting in comparable emission intensity from both components [135].

As an alternative to phospholipid micelles, QD constructs may also be encapsulated in amphiphilic polymers. Polyethyleneimine was used to encapsulate hydrophobic CdSe/CdS/ZnS QDs ($\lambda_{em} = 610$ nm) to give particles with a hydrodynamic diameter of ~100 nm that contained dozens of QDs. In order to furnish a functional oxygen sensor, a chloroform solution of QDs ($\lambda_{em} = 470$ nm) and $[\text{Ru}(\text{dpp})_3]^{2+}$ in a 1:3 ratio was treated with the polyethyleneimine polymer to give <100 nm particles. The two components are spectrally separated and do not interact via FRET. The construct exhibited a ratiometric response from 0–152 Torr O_2 (0–20 % O_2). These nanoprobes were used to measure oxygen concentrations in cells and tumor spheroids (*vide infra*) [136].

Biological applications

Despite the variety of construct designs and diversity of O_2 -responsive phosphorescent molecules, only a few of these sensors have been applied to study biological systems: *ex vivo* tissue samples, live cells, and animals. The CdSe/CdS QD and Pt(II) octaethylporphyrin ketone sensor dispersed in a PVC matrix was used for oxygen sensing in *ex vivo* brain tissue [125, 126]. First, a glass slide coated with the sensor was used to monitor oxygen levels in a murine hippocampal slice. The tissue exhibited a radial oxygen gradient from 18 mg/L (560 μM or 340 Torr) to 5 mg/L (160 μM or 100 Torr) at a distance of 250 μm from the edge of the sample, despite perfusing the tissue with gas containing 31 mg/L (970 μM or 590 Torr) O_2 during data acquisition. Next, the potassium channel blocker 4-aminopyridine was added to induce hyperexcitability. This resulted in spontaneous

seizure-like events (SLE) that caused a decrease of oxygen that was attributed to oxygen consumption in the tissue. The sensor was also coated onto a microelectrode, enabling simultaneous voltage and O_2 sensing in a small $25\ \mu\text{m} \times 25\ \mu\text{m}$ region of the hippocampus. These measurements also demonstrated a decrease in pO_2 during the spontaneous SLEs that resulted in a prolonged period of hypoxia that persisted for $>30\ \text{s}$ after completion of the SLE. This approach was further miniaturized to measure pO_2 using a fine glass electrode with the sensor matrix in conjunction with an intracellular electrode to measure these two parameters in a single cell, reducing the area to $3\ \mu\text{m} \times 3\ \mu\text{m}$ [126].

In contrast to solid-state devices, soluble oxygen sensors are more amenable for measuring pO_2 in live cells. Human colon cancer cells (HCT116) were treated with the polyethyleneimine-encapsulated QD/ $[\text{Ru}(\text{dpp})_3]^{2+}$ sensor and cultured under normoxic or hypoxic conditions [136]. It was found that the pO_2 in these samples was 20.5 % (156 Torr) and 1.1 % (8 Torr), respectively. Similarly, this sensor was used to study a tumor spheroid, which mimics metastatic tumors, using the HCT116 cell line. The spheroid exhibited a radial oxygen gradient with the core being most hypoxic (1.3 % or 10 Torr); pO_2 increased to 8.4 % (64 Torr) halfway from the center, and finally 19.9 % (151 Torr) at the periphery of the spheroid. Similarly, the QD/perylene sensor was used to measure oxygen concentrations in HeLa cells [128]. A 30 % increase in the perylene:QD emission ratio was observed for cells cultured overnight under 6.9 % (52 Torr) vs. 19.8 % (150 Torr) oxygen (Fig. 9a). The $\text{Ir}(\text{piq})_3/\text{QD}$ nanoparticle sensor embedded in a monooleoylglycerol matrix was also used to examine pO_2 of HeLa cells cultured under different oxygen tensions [124]. The $\text{Ir}(\text{piq})_3:\text{QD}$ emission ratio increased from 1.42 to 3.96 for cells cultured under 21 % (160 Torr) and 1 % (8 Torr) O_2 , respectively.

To date, there have been few reports of *in vivo* oxygen sensing with QD conjugates. In one experiment, HeLa cell tumor xenografts were implanted on the leg of nude mice. Oxygen levels, as measured by the $\text{Ir}(\text{piq})_3/\text{QD}$ sensor, in the tumor and muscle tissue were compared and it was found that the $\text{Ir}(\text{piq})_3:\text{QD}$ emission ratios were 1.79 and 0.93, respectively [124]. As expected, the tumor exhibited higher $\text{Ir}(\text{piq})_3$ phosphorescence and was more hypoxic than muscle tissue and the sensor was able to qualitatively differentiate these two tissues. However, differences between the emission ratios observed for *in vitro* calibration and *in vivo* measurements prevented the translation of these values into precise oxygen concentrations. Indeed, this remains an unsolved problem in the field. The identity of the tissue, as well as the depth at which the image is acquired, has a profound effect on the observed intensity ratio [137]. Optical properties, such as absorption and scattering coefficients, are tissue-dependent [138]. Consequently, the collection efficiency of photons from the QD and phosphorescent molecule may not be identical, resulting in differences between *in vivo* ratio measurements and *in vitro* calibrations [135]. One way to circumvent this limitation is to perform lifetime-based measurements, as this parameter is independent of the optical properties of the tissue. The micelle-encapsulated QD/Pd(II) porphyrin sensor was systemically injected into mice with surgically implanted cranial windows or dorsal skinfold chambers. Mice were anesthetized and subsequently imaged using two-photon microscopy ($\lambda_{\text{ex}} = 850\ \text{nm}$). Emission from the QD was homogenous throughout the vessels (Fig. 9b), while the porphyrin emission was more variable (Fig. 9c), reflecting differences in oxygenation of

Fig. 9: (a) Ratiometric response of the QD/perylene oxygen sensor in HeLa cells cultured under 19.8 % (150 Torr) or 6.9 % (52 Torr) O_2 . Reprinted with permission from [128]. Copyright 2016 American Chemical Society. Three-dimensional depth projections of murine brain vasculature under two-photon excitation ($\lambda_{\text{ex}} = 850\ \text{nm}$), showing the (b) QD and (c) Pd(II) porphyrin emission channels. Reprinted with permission from [133]. Copyright 2015 American Chemical Society.

different vessel types. In order to obtain quantitative information, lifetime measurements were acquired at various points and converted to oxygen concentrations using Eq. 1 with k_q from *in vitro* calibrations and τ_0 measured under two-photon excitation. As a result, quantitative oxygen concentrations were measured for arteries (51–72 Torr), veins (20–30 Torr), and capillaries (22–29 Torr) that are consistent with previous observations measured by phosphorescence quenching [139, 140]. This study demonstrates that the micelle construct is a viable sensor for *in vivo* imaging and sensing using lifetime-based measurements [133].

Conclusions and outlook

Fluorescent semiconductor QDs possess exceptional properties that render them ideal scaffolds for the construction of optical sensors. They have multifaceted roles as internal intensity standards for ratiometric sensing, as well as FRET donors and multiphoton antennas in both solid-state and solution-phase sensors. Solid-state sensors in polymer matrices can be coated on a variety of substrates or dispersed into micro- or nanoparticle formulations. Soluble sensors may be prepared by covalent conjugation, self-assembly, or micelle encapsulation. The latter two methodologies represent facile, rapid, and scalable processes that enable the preparation of constructs with precise donor–acceptor ratios. The direct binding of acceptor molecules to the QD surface results in conjugates with high FRET efficiency. Several sensors have been used for a variety of biological applications, measuring pO_2 in *ex vivo* tissue samples, cells, and live animals. Moreover, the various methodologies for preparing QD-based conjugates can be generalized to the study of other biomedically-relevant analytes, such as nitric oxide, Ca(II) ions, and oncometabolites.

Ideally, these ratiometric sensors could be used to determine oxygen concentrations based on *in vivo* images alone, circumventing the need for lifetime-based measurements. This would enable faster data acquisition and provide greater fidelity in rapidly detecting real-time changes of pO_2 in live animals. However, ratios measured *in vivo* cannot translate to meaningful oxygen concentrations due to differences in the collection efficiency of QD and phosphorescent molecule emission, thereby skewing the measured ratios (*vide supra*). To overcome this limitation, a robust, reliable methodology for data acquisition and processing must be developed. Calibrations can be performed in tissue phantoms or *ex vivo* tissue samples to experimentally measure differences in the collection efficiency of the two sensor components as a function of imaging depth. However, such calibrations are tissue-specific and may not accurately recapitulate the complexity of real *in vivo* samples. Complementary Monte Carlo simulations may be necessary to model photon scattering and assist in obtaining meaningful ratios. Alternatively, shifting the emission profiles of both the QD and phosphorescent molecule into the red and NIR regions of the spectrum will minimize differences in photon scatter. This necessitates the development of novel red- and NIR-absorbing optical oxygen sensors, as traditional phosphorescent molecules absorb in the blue or green region of the spectrum. A recent strategy to impart oxygen sensitivity to QD photoluminescence is through the direct binding of carboxylic acid-modified PAHs (e.g. anthracene and pyrene) to the surface of a QD [141–143]. In these systems, Dexter-like triplet energy transfer occurs between the PAH and QD, resulting in long-lived (0.3–50 ms) QD photoluminescence. In principle, this phenomenon can be used for optical oxygen sensing; the inclusion of an additional, oxygen-invariant fluorophore would render a ratiometric construct.

One concern with the use of QD-based sensors is their potential toxicity. It has been demonstrated that overcoating with an inert shell (e.g. ZnS or SiO₂) significantly decreases toxicity, although long-term leaching of Cd(II) ions from the core remains underexplored. Other aspects of toxicity include colloidal instability and the generation of ROS [31, 144]. An alternative approach is to use QDs that do not contain heavy metals [145]. In any case, the primary utility of QD-based sensors is to study human disease in animal models. For example, patient-derived tumor xenografts can be implanted in a mouse to measure functional parameters for tumor growth and response to therapy, including pO_2 and pH. This is an inroad to personalized medicine, developing patient-specific therapeutic regimens. The continued development of QD-based sensors will provide novel tools for oncologists and clinicians to understand fundamental aspects of tumor biology and develop novel pharmaceuticals and therapeutic protocols.

Acknowledgements: I would like to thank Prof. Daniel G. Nocera for his guidance and support during my graduate studies. I also thank Prof. Michael A. Marletta for mentorship during my postdoctoral training. Nancy Li, Benjamin G. Horst, Dr. John A. Hangasky, and Dr. Andrew G. Maher are thanked for critical input during the preparation of this manuscript. C.M.L acknowledges the Miller Institute for Basic Research in Science at the University of California, Berkeley for a postdoctoral fellowship.

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