

Click-Assembled, Oxygen-Sensing Nanoconjugates for Depth-Resolved, Near-Infrared Imaging in a 3D Cancer Model**

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Abstract: Hypoxia is an important contributing factor to the development of drug-resistant cancer, yet few nonperturbative tools exist for studying oxygenation in tissues. While progress has been made in the development of chemical probes for optical oxygen mapping, penetration of such molecules into poorly perfused or avascular tumor regions remains problematic. A click-assembled oxygen-sensing (CAOS) nanoconjugate is reported and its properties demonstrated in an in vitro 3D spheroid cancer model. The synthesis relies on the sequential click-based ligation of poly(amidoamine)-like subunits for rapid assembly. Near-infrared confocal phosphorescence microscopy was used to demonstrate the ability of the CAOS nanoconjugates to penetrate hundreds of micrometers into spheroids within hours and to show their sensitivity to oxygen changes throughout the nodule. This proof-of-concept study demonstrates a modular approach that is readily extensible to a wide variety of oxygen and cellular sensors for depth-resolved imaging in tissue and tissue models.

Cancerous lesions are host to heterogeneous microenvironments that vary in their properties and response to drugs.^[1,2] In many cancers, including ovarian cancer (OvCa), drug resistance has been linked to the presence of hypoxia, which has been shown to reduce therapeutic efficacy through a variety of mechanisms.^[1]

Overcoming this hypoxia-induced treatment resistance requires model systems capable of recapitulating the complex tumor environment observed in human disease. In vitro tumor models, such as OvCa 3D spheroid cultures, exhibit many of the features of in vivo metastatic lesions, such as the

presence of a hypoxic core, while also providing an environment that allows for consistent imaging at the same location before, during, and after therapy.^[2–4] This approach enables a cell-by-cell correlation of microenvironmental factors and therapeutic response. The ability to incorporate hypoxia imaging into this platform, however, has been hampered by the absence of cellular-resolution oxygen-sensing methods capable of providing depth-resolved imaging in in vitro model systems. Phosphorescence lifetime imaging is based on oxygen-dependent quenching of phosphorescence and is a technique well-suited for the study of spheroid oxygenation.^[5] In this approach, the phosphorescence decay time of an exogenous chemical oxygen sensor is measured and related to oxygen concentration through a Stern–Volmer-type relationship.^[6] This technique, developed in parallel with novel molecular oxygen sensors such as Oxyphor G2, has been used to quantify in vivo oxygenation in a variety of tissue types, including tumor, brain, retina, and heart, and has been deployed using numerous modalities.^[6–8] However, nonperturbative measurements of intracellular oxygen tension deep within tissue have not yet been achieved and methods for delivering oxygen sensors through hundreds of micrometers of cellular layers to hypoxic tumor regions have not yet been developed. Moreover, the molecular oxygen probe Oxyphor G2, while well-characterized and commercially available, is incapable of spontaneously penetrating into cells or multicellular structures (Figure S1).^[9]

This work describes the development of click-assembled oxygen-sensing (CAOS) nanoconjugates comprising novel poly(amidoamine)-like dendrons and demonstrates their spheroid-penetrating abilities.^[3,4] These nanoconjugates combine near-infrared (NIR) emissivity, lack of toxicity at functional doses, and the ability to spontaneously penetrate hundreds of micrometers into 3D tumor models within hours. These properties make the CAOS nanoconjugates unique among oxygen sensors and enables oxygen-sensitive imaging at greater than 100 μm depths through the use of NIR-optimized confocal microscopy. Furthermore, our modular synthetic approach enables the synthesis of higher generation dendrons within days instead of weeks. The ability of the CAOS nanoconjugates to spontaneously penetrate multiple cellular layers overcomes a key barrier to the use of in vitro tumor models as hypoxia model systems, while also enabling a host of other applications such as oxygen-sensitive imaging in poorly- or non-vascularized tissue regions.

Many oxygen sensors are dendrimeric structures, in which an oxygen-sensitive porphyrin core is surrounded by dendritic branches, or dendrons. In protein-free solutions, these dendrons serve to both solubilize the central porphyrin and modulate its dynamic range of oxygen sensitivity.^[6] Import-

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tantly, in protein-rich environments such as the bloodstream, non-PEGylated dendrimers have been shown to interact extensively with endogenous proteins; this interaction can decrease the rate of oxygen quenching by an order of magnitude or more.^[6] We have observed that fourth generation dye-tagged poly(amido) amine (G4 PAMAM) “starburst” dendrimers were taken up by monolayer OvCa cultures after 40 min (Figure S2); the same molecules penetrated throughout OvCa 3D spheroids of greater than 250 μm diameter after 3.5 h at low concentrations (500 nM) with no observable toxicity (Figure S3, Movie S1 in the Supporting Information). PAMAM dendrimers are known to form complexes with proteins such as bovine serum albumin (BSA) and are readily endocytosed.^[10,11] We therefore reasoned that a phosphorescent porphyrin coupled to one or more higher-generation (\geq G2) PAMAM dendrons would benefit from the cell- and spheroid-penetrating properties of PAMAM while also interacting with cellular proteins, thereby creating an oxygen sensor with a useful dynamic range for biological experiments.^[10,11] For this work, we selected palladium tetracarboxytetrabenzoporphyrin (PdTCTBP) as our phosphorescent porphyrin core because its lifetime properties have been characterized in the literature, its synthesis is well-known, and its NIR emission (810 nm) lies in the “optical window” ideal for tissue imaging. Additionally, the spectral profile of PdTCTBP minimizes overlap with known regions of intense autofluorescence and it can be excited by readily available laser lines (445 nm and 635 nm).^[6]

Prior synthetic schemes involving synthetic modification of commercially available PAMAM dendrons followed by convergent assembly around an oxygen-sensitive porphyrin resulted in low yields. Therefore, to produce the spheroid-permeable oxygen sensor described herein, we developed a click-based strategy to rapidly assemble PAMAM-like subunits and couple them to an oxygen-sensitive porphyrin core. PAMAM dendrons with azide/alkyne functionality have been reported in the literature but they problematically make use of extremely slow reactions and require months to synthesize.^[12] In contrast, our scheme enables the rapid assembly of PAMAM-like dendrons from three branched subunits (Figure 1a and Figure S5). Briefly, the synthetic scheme relies on the differing labilities of triethylsilyl (TES) and triisopropylsilyl (TIPS) alkyne-protecting groups, which allows sequential subunit ligations while avoiding polymerization (Figure 1b, Figure S6). First, the TIPS-protected “starting” subunit **1d** is reacted with 2 equivalents of the “intermediate” subunit **2c** and catalytic quantities of sodium ascorbate and CuSO_4 . Following isolation of the reaction product **4a**, the TES protecting groups are removed by using mild conditions that leave the focal-point TIPS protecting group intact, thereby yielding **4b** and preparing the growing dendron for another round of ligation. Primary amine functionality can then be added by ligation of the “final” subunit **3b** to create **4c**, the focal-point alkyne of which is then deprotected by using tetrabutylammonium fluoride to yield **4d**.^[13] Each dendron growth phase can be conducted overnight, and the product can be isolated without the use of chromatography. This allows higher-generation triazole-bridged PAMAM-like dendrons to be synthesized in

a matter of days as opposed to months. Once complete, the dendrons can be conjugated to any central moiety in a single convergent step. In this study, we coupled the dendrons to palladium tetraazidotetrabenzoporphyrin (**5b**), an azido-functionalized PdTCTBP derivative, to give the CAOS nanoconjugate **6a**, which was then deprotected to give **6b** (Figure 1c).

By using this sequential click method, we synthesized third-generation (G3) CAOS nanoconjugates from G2 PAMAM-like dendrons. The palladium tetraazidotetrabenzoporphyrin core is capable of reacting with up to four equivalents of dendrons. In practice, a distribution of adducts was observed. The G3 CAOS nanoconjugates were characterized by NMR and MALDI-TOF MS as being composed of mono-, di- and tri-dendron adducts; NMR integration suggests that, on average, between two and three dendrons are attached to each nanoconjugate (Figures S8, S29–31).

The G3 CAOS nanoconjugates were studied in a dense, protein-rich environment and were found to exhibit uniform oxygen sensitivity. To evaluate their photophysical properties, we dissolved the nanoconjugates in 2% bovine serum albumin (BSA) and monitored their phosphorescence lifetime as a function of pO_2 .^[6] Oxygen quenching of phosphorescence is traditionally described using the Stern–Volmer relationship [Eq. (1)]:

$$\frac{1}{\tau} = k_q [\text{pO}_2] + \frac{1}{\tau_0} \quad (1)$$

Where τ is the exponential lifetime of the phosphor, k_q is the quenching coefficient, $[\text{pO}_2]$ is the partial pressure of dissolved oxygen, and τ_0 is the exponential lifetime of the phosphor in the absence of oxygen.^[6] k_q is a function of the chemical environment of the phosphor and reflects the sensor’s dynamic range; in general, a lower k_q value is desirable. We compared the k_q and τ_0 values of the G3 CAOS nanoconjugates to those of Oxyphor G2 (Figure 2, Table 1), which possesses a PdTCTBP core surrounded by second generation glutamate dendrimers.^[6,7] The order of magnitude of the k_q value observed for the G3 CAOS nanoconjugates is consistent with that of Oxyphor G2, the calibration is linear, and the dynamic range is sufficient for oxygen measurement across the physiological range.^[6]

Table 1: Calibration constants for the G3 CAOS probe and Oxyphor G2.

	τ_0 [μs] ^[a]	k_q [$\text{mmHg}^{-1} \text{s}^{-1}$] ^[b]
G3 CAOS probe	173	377
Oxyphor G2	178	283

[a] τ_0 = phosphorescence lifetime in zero oxygen.

[b] k_q = phosphorescence quenching constant.

Having verified that the G3 CAOS nanoconjugates exhibit the desired photophysical properties, we next tested their uptake in cells. Given the low quantum yield ($< 1\%$) of PdTCTBPs in ambient oxygen conditions and the low NIR throughput and sensitivity of commercially available micro-

scopes, we first tagged the G3 CAOS nanoconjugates with the fluorophore AlexaFluor 488. Confocal imaging of OvCa cells treated with G3 CAOS probe conjugated to AlexaFluor 488

(500 nm G3, 40 min) confirmed cellular uptake and revealed a punctate subcellular pattern consistent with lysosomal localization (Figure S35), as described in previous reports.^[11]

An MTT viability assay confirmed an absence of toxicity at the time of imaging at all doses used (1 μM –10 μM , Figure S4).

To image the weak, oxygen-dependent phosphorescence of the G3 CAOS nanoconjugates, we built a single-photon-counting confocal microscope and optimized its performance for imaging in the 800–900 nm wavelength range that corresponds to the emission of the G3 CAOS nanoconjugates (Figure S34). We used this system to confocally image OvCa cells treated with unlabeled G3 CAOS nanoconjugates, thereby demonstrating the successful creation of a cell-penetrating oxygen-sensitive nanoconjugate (Figure S35). We also observed an inverse dependence of the phosphorescence intensity on $p\text{O}_2$: there was a two- to four-fold increase in signal following a purge of the cell culture dish with nitrogen.

Using the photon-counting NIR confocal microscope, we confirmed that the G3 CAOS nanoconjugates penetrate 3D OvCa spheroids. Prior to imaging, the spheroids were incubated with 2 μM G3 CAOS nanoconjugates for four hours. 3D volume acquisition with confocal optical sectioning revealed that the phosphorescence signal could be observed throughout each spheroid at depths exceeding 100 μm , whereas no signal was observed in untreated spheroids (Figure 3, Movie S2). To confirm the probe's sensitivity to oxygen levels, we purged the culture dish with nitrogen and observed an approximate doubling of the phosphores-

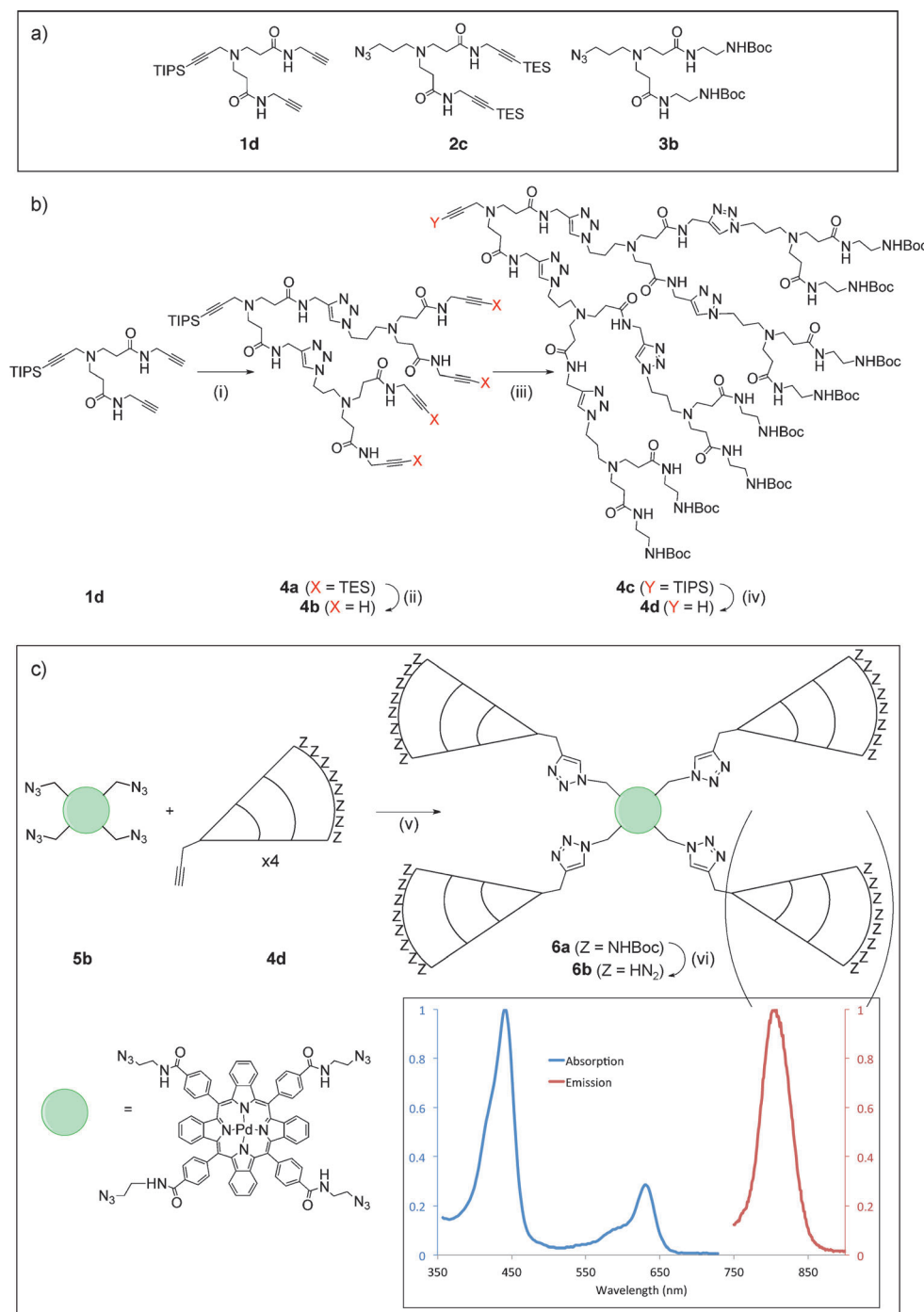


Figure 1. Generalized overview of the synthetic scheme used in the synthesis of the CAOS nanoconjugates. a) The dendron subunits used in the assembly of the PAMAM-like dendron. The TES and TIPS protecting groups differ in their lability. b) Synthesis of G3 PAMAM-like dendrons through the sequential click ligation of subunits. i) **1d** (1 equiv), **2c** (2 equiv), CuSO₄ (0.2 equiv), sodium ascorbate (0.4 equiv), DMF/H₂O, RT; ii) K₂CO₃ (50 equiv), MeOH, 36 h, RT; iii) **4b** (1 equiv), **3b** (4 equiv), CuSO₄ (0.2 equiv), sodium ascorbate (0.4 equiv), DMF/H₂O, RT; iv) TBAF (5 equiv), THF, RT. c) An overview of the convergent nanoconjugate assembly step. v) **5b** (1 equiv), **4d** (5 equiv), CuSO₄ (12 equiv), sodium ascorbate (24 equiv), DMF/H₂O, RT; vi) TFA, dichloromethane, RT. Inset: Normalized absorption and emission spectra of the G3 CAOS nanoconjugates. DMF = *N,N*-dimethylformamide, TBAF = tetra-*n*-butylammonium fluoride, THF = tetrahydrofuran, TFA = trifluoroacetic acid.

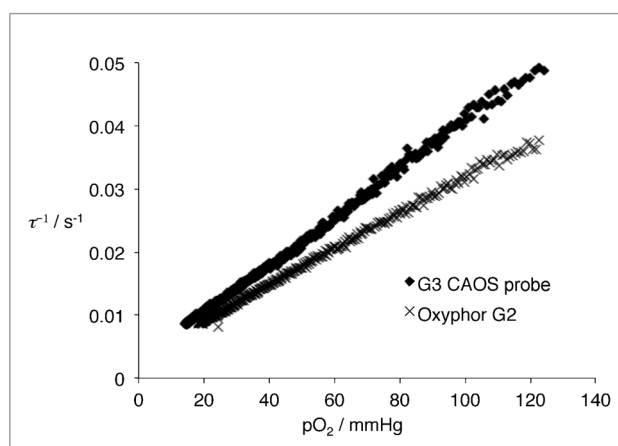


Figure 2. Calibration data for the G3 CAOS probe and the commercially available Oxyphor G2. Measurements were performed in a 2% BSA solution. τ = phosphorescence lifetime (s).

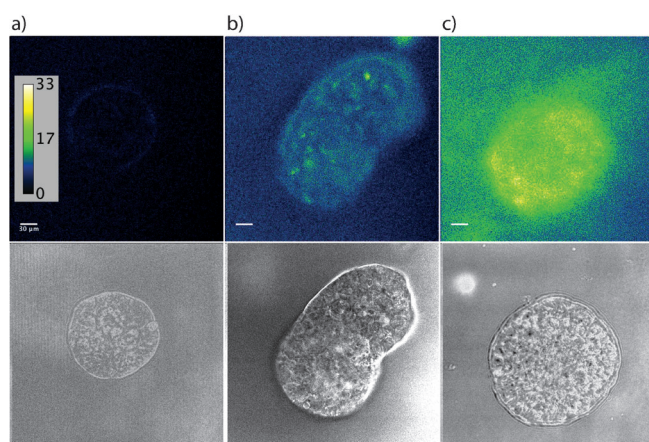


Figure 3. NIR phosphorescence confocal microscopy imaging reveals that G3 CAOS probes spontaneously penetrate 3D OvCa spheroids. a) 3D OvCa spheroids, no treatment control (bottom/gray panels: transmission channel). b) False-color image of spheroids treated for 4 h with 2 μm G3 CAOS nanoconjugates, imaged under air. c) Spheroids treated for 4 h and imaged after a nitrogen purge. All images were acquired at the approximate midpoint along the z-axis of each spheroid, a point that corresponds to an approximate depth of 100 μm . Legend bar: phosphorescence photon count, scale bar: 30 μm .

cence signal arising from the spheroids. Different spheroids were imaged before and after the nitrogen purge to avoid the influence of photobleaching.

In conclusion, we have developed a novel approach for the synthesis of oxygen-sensitive nanoconjugates and provide

proof-of-concept that click-assembled nanoconjugates can be used as deep tissue imaging agents for 3D oxygen-sensitive imaging. Our assembly strategy does not require the long reaction times typically necessary to achieve higher generation dendrons. Future work in this area will involve the synthesis and testing of a ratiometric CAOS nanoconjugate, which would facilitate more straightforward calibration and quantitative oxygen mapping in the complex cellular environment. Additionally, we chose PdTCTBP for an initial demonstration of the CAOS approach because of the extensive body of work describing its use for quantitative oxygen measurements in biological systems;^[6] future work will make use of phosphorescent porphyrins with a higher quantum yield, thereby enabling imaging on less specialized microscopes and reducing the barrier to the use of CAOS-type nanoconjugates in biological experiments.

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