Biodegradable and Switchable Near-Infrared Fluorescent Probes for Hypoxia Detection

*Indrajit Srivastava,[1,2] Parikshit Moitra,[3] Kurtis M. Brent, [1,2] Kevin Wang, [1,2] Subhendu Pandit, [1,2] Esra Altun,[1,2] Dipanjan Pan[1,2,3,4,5,]**

[1]Department of Bioengineering, University of Illinois at Urbana-Champaign, Urbana, IL, 61801, USA

[2] Mills Breast Cancer Institute, Carle Foundation Hospital, Urbana, IL, 61801, USA

[3]Department of Nuclear Engineering, The Pennsylvania State University, University Park, PA 16802, USA

[4]Department of Materials Science and Engineering, The Pennsylvania State University, University Park, PA 16802, USA

[5]Huck Institutes of the Life Sciences, 101 Huck Life Sciences Building, University Park, PA 16802, USA

Keywords. hypoxia, chemotherapeutic resistance, oxygen level, carbon dots, near-infrared emission, reversible fluorescence, bioimaging, biodegradable, digestive enzyme, ex-vivo.

Abstract.

Aims: Among solid tumors, hypoxia is a common characteristic and responsible for chemotherapeutic resistance. Hypoxia-sensitive imaging probes are therefore essential for early tumor detection, growth monitoring, and drug response evaluation. Despite significant efforts, detecting hypoxic oxygen levels remain challenging. **Materials & Methods:** This paper demonstrates the use of an amine-rich carbon dot probe functionalized with an imidazole group that exhibits reversible fluorescence switching in normoxic and hypoxic environments. **Results and Conclusions:** We demonstrate the ability to emit near-infrared light only under hypoxic conditions. The probes are found to be biodegradable in the presence of human digestive enzymes such as lipase. Ex-vivo tissue imaging experiments revealed promising NIR signals even at a depth of 5 mm for the probe under ex-vivo imaging conditions.

Plain Language Summary. Hypoxia is the state where oxygen is not adequately available at the tissue level and is the common cause of resistance towards chemotherapeutics. Hence, probes those can detect hypoxia are important in detecting early tumor progression. Here in this paper, we have developed a fluorescent probe which helps in determining normoxic and hypoxic environments. This probe emits near-infrared light only under hypoxic conditions. The phenomena have been established herein by extensive experiments.

Introduction

The early detection of tumors has become an increasingly important area of focus today. [1]. There is a characteristic feature of solid tumors that the oxygen concentration is relatively low. This is caused by the structure and function of the microvessels supplying nutrients to the tumor. [2]. The same holds true for tumors as small as a few millimeters in diameter [3]. Therefore, targeting this trait of solid tumors provides promise for early cancer detection. Hypoxia is a condition where the tissue environment is deprived of adequate oxygen supply. Aside from cancerous tumors, it is often a common indicator of other diseases including cardiovascular diseases [4]. Hypoxia is not only critical for early detection of solid tumors, but it is also a sign of resistance to chemotherapy [5]. It is therefore essential to be able to detect hypoxia early through non-invasive, sensitive imaging methods. Currently, the primary technique for hypoxia detection is to use an oxygen-sensitive electrode. Despite high accuracy of this electrode, it is disfavored because it is highly invasive [5]. Other studies have considered the use of positron emission tomography and single photon emission computed tomography to image hypoxia markers labelled with radiotracers [6]. However, specific, and accurate detection of hypoxia can be difficult with this method due to the very small oxygen concentration necessary from the build-up of these probes at the tumor site [7]. Conversely, for the detection of hypoxia *in vivo*, several fluorescent based probes have been utilized over the last few years [8-14]. Hypoxia is often accompanied by overexpression of nitroreductase (NTR) enzyme, detection of which has been targeted by numerous chemically modified probes that emit near-infrared emission in presence of the NTR enzyme. Atkinson et al. developed a new synthetic strategy to create bioresponsive NIR probes that are hydrophilic and in presence of NTR enzyme can undergo cleavage followed by enhancement of fluorescence signals [11]. Recently, Zhao et al. developed stimuli-responsive

fluorescent probes that have improved specificity and multiplexed capability of detecting hypoxia and glutathione (found in high concentrations in tumor cells) [9]. However, some of these probes, such as modified indocyanine green to detect hypoxia, have suffered from low quantum yield and rapid fluorescence lost [15].

In this work, we have utilized carbon dots (CDs) and synthesized hypoxia-sensitive probes that specifically exhibited strong fluorescence in the near-infrared (NIR) region in a hypoxic environment. Our choice of CDs was attributed to their high photostability, high water solubility, and low cytotoxicity [8]. The probes were designed by taking amine-rich CDs (pCD) with quenched fluorescence in the NIR region and then functionalizing it with a nitroimidazole-based small molecule. These amine rich CDs were synthesized using *para*-phenylenediamine as a precursor via solvothermal process as represented in **Figure 1**. *Compared to conventional fluorescent probes, these CDots-based probes for detecting hypoxia have numerous advantages. This includes simple synthesis steps, high water solubility, activable fluorescence and high signal stability in hypoxic environments, biodegradable, and potential to be combined with cancer therapies as the probe generates oxidative stress when activated under hypoxic tumor environments.*

Adding a nitroimidazole group to pCD has the advantage of engaging in a nucleophilic covalent bond with proteins commonly found in low-oxygen environments [16-24]. They are often used for hypoxic detection because nitrogen dioxide is reversibly reduced to an amine under hypoxic environments [17]. As amine groups on pCD can be further functionalized with epoxy groups, 1-(2, 3-epoxypropyl)-2-nitroimidazole (NIDz) is used herein to create oxygen-sensitive pCDs. These probes had quenched fluorescence in a normoxic environment, however, once subjected to a hypoxic environment (<1% pO_2), they exhibited strong NIR fluorescence (switch-

on) with high sensitivity, selectivity, and photostability. A reversible aspect of this phenomenon was found as well, since the probe lost its fluorescence when exposed to normoxia following hypoxia. (**Figure 1**).

Methods

Materials.

p-Phenylenediamine ($M_w \approx 108$) and 1-(2,3-epoxypropyl)-2-nitroimidazole ($M_w \approx 169$) were both purchased from Sigma-Aldrich (MO, USA). Ninhydrin ($M_w \approx 178$) was also purchased from Sigma-Aldrich. L-Phenylalanine ($M_w \approx 165$) was purchased from Chem-Impex (IL, USA). The ROS-ID Hypoxia/Oxidative stress detection kit was purchased from Enzo Life Sciences. Lipase from human pancreas and hemoglobin were both purchased from Sigma-Aldrich.

Fabrication of the carbon dot probe.

pCD was synthesized by dissolving 200 mg of *p*-phenylenediamine in 10 mL of ethanol. The solution was then placed in an autoclave for 6 hours at 180 ˚C. Following this, another 10 mL of ethanol was added to the product. The solution was then sonicated and filtered into a new vial. The solvent was evaporated using a rotary evaporator to isolate the pCD. Finally, sufficient nanopure water (0.2 mM, 18 MΩ) was added to the pCD to attain a final concentration of 5 mg/mL. This was determined to be the optimum pCD concentration from our previous experience [20-35].

Functionalization of the probe with NIDz.

Following the fabrication of the probe, NIDz was added and vortexed with pCD to generate the

functionalized carbon dot probe (pCD-Norm). A vacuum desiccator (<1% pO_2) was used to simulate the hypoxic environment and test our probe in hypoxia (pCD-Hyp). During functionalization, equivalent volumes of pCD and NIDz were mixed. Following the optimization steps, characterization techniques including Fourier-transform infrared spectroscopy and X-ray photoelectron spectroscopy were done to study the surface chemistry of the synthesized probe [36,37]. Mass spectrometry was used to confirm the molecular structures of the probes [38].

Dynamic light scattering measurements.

Dynamic light scattering (DLS) measurements were conducted to measure the hydrodynamic size distribution (intensity average) of the NPs on Malvern Zetasizer ZS90 instrument (Malvern Instruments Ltd., United Kingdom) at a fixed angle of 90°.

UV-Vis and fluorescence spectroscopy.

UV-Vis and fluorescence spectroscopy were both taken using a Synergy HT plate reader (BioTek, VT, USA). Absorbance spectra of probe samples were obtained by scanning from 300-800 nm with an interval of 5 nm. Fluorescence spectra were acquired at excitation wavelength of 465 nm. Fluorescence spectroscopy was also recorded from a NanoDrop 3300 fluorospectrometer (Thermo Scientific, USA) using an excitation wavelength of 475 nm [39].

Gel dock luminescence imaging.

Gel dock luminescence images of our samples were obtained from a BioRad ChemiDock gel analysis instrument [17]. A Cy5 dye red channel was used with approximate excitation and

emission wavelengths of 647 and 690 nm respectively.

Quantum yield measurements.

The quantum yield of CDots in normoxia and hypoxia environment was tested using an integrating sphere (IS200-4, Thor Labs) that was attached to spectrofluorometer (RF-6000, Shimadzu). Firstly, solution of CDots subjected in normoxic and hypoxic environments were diluted such that their absorption intensity were below 0.1 and the excitation wavelength of 475nm. Then, the CDots solutions were added to the fluorescent cuvette and the fluorescent spectra were recorded from 500n-700nm. As a control, fluorescent spectra of deionized water were recorded under identical experimental conditions. Finally, the fluorescent software was utilized to calculate the quantum yields.

Ninhydrin assay.

Absorbance measurements of the samples were taken with a Synergy HT plate reader (BioTek, VT, USA) at 570 nm. Ninhydrin was first dissolved in acetone and then added to the samples of L-phenylalanine with known concentrations [15]. A ninhydrin assay of L-phenylalanine was first performed to acquire a linear function of amines in a sample. After conducting a ninhydrin assay with samples of our probe, the concentration of free amines was calculated using the generated linear function from the L-phenylalanine experiments. The absorbance values from the probe experiments were obtained after subtracting the absorbance value from a blank. Within this context, free amines refer to the amines on pCD that are not functionalized with NIDz. The percent surface coverage of NIDz was found by simply subtracting the percent of free amines from the

total amines.

Fluorescence imaging.

Fluorescence imaging was accomplished using a PerkinElmer IVIS Spectrum CT live-animal imaging system [18]. Epi-fluorescence measurements were taken of our imaged *ex vivo* tissue.

Transmission electron microscopy.

TEM images were recorded on JEOL 2010 LaB6 operating at 200kV.

Electron paramagnetic resonance.

Electron paramagnetic resonance data was collected from a Bruker 10" EMXPlus X-band Continuous Wave EPR. Samples of pCD, pCD-Norm and pCD-Hyp were tested for the presence of radical species [18]. Before testing the hypoxic response of our probe, we tested for the presence of reactive oxygen species that could induce oxidative stress on cells.

MTT Assay.

MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)) was purchased from Sigma-Aldrich. The cytotoxic effects of various samples in two different cell lines (MCF7 and MDA-MB231) were investigated using MTT assay. MTT was added to samples of 10,000 cancer cells incubated with varying concentrations of pCD-Hyp in a 96 well-plate for 48 hours [40]. After 4-hour incubation of the samples with MTT, DMSO was added to dissolve the formazan crystals

and a Synergy HT plate reader (BioTeK, VT, USA) was used to record absorbance values.

Flow cytometry.

Flow cytometry data of cells was attained from a Guava EasyCyte Plus Flow Cytometer. MCF-7 cells were cultured and examined for the presence of hypoxia and oxidative stress after treatment with our probe. The detection assay kit, we used, provided reagents for measuring both oxidative stress and hypoxia where the oxidative stress detection reagent was responsive in the green channel and the hypoxia detection reagent was responsive in the red channel. We cultured 8 samples of MCF-7 cells and mixed all of them with a detection mix that contained both detection reagents. Following treatment of the cells with their respective agents, 4 of the samples were measured after 0.5 hours for oxidative stress. This is because the positive control agent, pyocyanin, generates oxidative stress around this time which gradually dissipates afterwards. We used FlowJo software to corner as many cells of our control sample to one region of the data as the cells here should not be responsive to the green or red channel. We maintained these parameters for the other 3 samples for oxidative stress. We performed another experiment where we treated the cells with pCD in order to compare as a control to pCD-Hyp. We repeated the same protocol for hypoxia, except using deferoxamine as the positive control that induces hypoxia after ~3.5 hours. We plotted our data to place our target cells response on the x-axis, thus positioning our quadrants of interest on the right side of the graph (Q2 and Q3).

Biodegradability with human lipase.

200 µL of human lipase (1mg/mL) was mixed with 100 µL of our functionalized probe and incubated at 37 ˚C in a dark environment. 100 µL of hydrogen peroxide (30%) was also added to commence the degradation of the probe by the enzyme. An additional 100 µL of hydrogen

peroxide was added after every 12 hours during incubation to maintain the enzymatic activity. Following incubation, the samples were submitted for mass spectrometric analyses.

Results

After synthesizing the pCDs, we first characterized their particle size distribution with dynamic light scattering (DLS) and found their hydrodynamic diameter to be ~17 nm (Fig. S1). From transmission electron (Fig. 2a) and atomic force microscopy (Fig. S2), the anhydrous size of the nanoparticles was found to be ~10 nm (as observed from TEM) with height of the nanoparticles ~100 nm (as observed from AFM, Fig. S2). We began with experiments to examine the efficacy of our probe. Electron paramagnetic spectroscopy (EPR) was also conducted to show the increase in generation of nitroxide free radical upon the NIDz functionalization and subsequent incubation in a hypoxic environment (Fig. 2b, Fig. S3). Figure 2c shows the UV-Vis absorption data of pCD, pCD-Norm and pCD-Hyp. In hypoxic environments, the probe shows a redshift and an increase in absorbance. Fig. 2d displays the fluorescence emission of the probe in normoxia and hypoxia when excited at 465 nm. pCD-Hyp displayed stronger fluorescent signals from 600 nm to over 700 nm relative to pCD-Norm and pCD. Further quantitative analysis on assessing the degree of hypoxia could be made using such CDots-based fluorescent probes (Fig. S4).

We attempted to evaluate the fluorescent quantum yields of pCD-Norm and pCD-Hyp using an integrating sphere connected to a fluorescence spectrometer. As expected, pCD-Norm had low quantum yield value ($\emptyset = 0.05$) whereas once it was subjected to a hypoxic environment, both fluorescent intensity and quantum yield increases ($\emptyset = 0.11$).

The next step was to optimize the chemical concentrations for synthesizing our probe. Previously acquired data has supported the optimal concentration of pCD as 5 mg/mL. However, Figure 2e provides qualitative optimization data via gel dock images of pCD-Norm and pCD-Hyp with increasing concentrations of NIDz. Greater fluorescent signals were obtained when using higher concentrations of NIDz. To acquire a quantitative measure for optimizing NIDz concentration, we performed a ninhydrin assay [41-42]. The figure 2f illustrates the results of this experiment, where we calculated the percent surface coverage of our carbon dot with NIDz by comparing UV-Visible absorbance values of functionalized and pure pCD samples. The sample with 50 mM NIDz was found to have the highest percentage of NIDz functionalized to pCD.

Our next step is to demonstrate that the NIR emission from pCD-Hyp can be attributed to the reduction of nitro groups on NIDz to amine groups when the nanoparticles are incubated with human hemoglobin. Patterson et. al. demonstrated that N-oxides could undergo two-electron reduction process via cytochrome P450 with a heme-iron centre as an active site [40]. In support of the hypothesis, we incubated normal human haemoglobin with heme-iron center in pCD-Norm (just functionalized and quenched probe). It is intended to evaluate whether it reduces the nitro group on NIDz functionality on pCD-Norm, resulting in a higher emission intensity. We found that, as Hb concentration increased to pCD-Norm, absorbance and fluorescence increased. 100 μM of Hb in 1:1 v/v ratio with pCD-Norm caused the maximum increase in absorbance and fluorescence intensity, thereby proving the reduction of nitro group on NIDz the most (Figure 3).

Following the proof-of-concept and optimization experiments, we proceeded to obtain preliminary results with *ex vivo* tissues to study the probe's success within biological environment [43-46]. Figure 4a presents the experimental data acquired from IVIS imaging. Figures 4b demonstrates different excitation wavelengths to optimize our settings whilst using IVIS

qualitatively and quantitatively. Samples of 5 mg/mL pCD with 50 mM NIDz were measured at different excitation wavelengths. Region of interest (ROI) measurements was taken from the samples and radiant efficiency was calculated as a measure of fluorescence emission radiance per incident excitation power. Excitation and emission values were found to successfully detect signals from pCD-Hyp at 640 nm and 720 nm respectively compared to pCD-Norm with higher intensity (Figure 4d). These settings were used for the remainder of the IVIS experiments. For the following IVIS experiments, raw chicken breast was imaged as our *ex vivo* tissue. Figure 4a depicts our method of gathering the *ex vivo* data with IVIS. Figure 4c displays IVIS imaging of the chicken breast tissues with 20 µL of the probe, previously in hypoxia, injected 2.5 and 5 mm deep. Signals detected at 2.5 mm were stronger, although sufficient signals were still detected at 5 mm depth for imaging. As time progressed, signals at both depths gradually diminished (Figure 4e). Figure 4f shows signal strength at various depths into the tissue immediately after probe injection. Signals closer to the surface were found to be stronger. The result has been presented in histograms in Figure 4g.

Figure 5 presents our *in vitro* cell data. Figures 5a and 5b show our MTT assay data from two different cell lines. MCF-7 cells showed lower cell viability and lower IC_{50} value compared to MDA-MB231 cells when treated with our probe. In figure 5c, we tested MCF-7 cells for oxidative stress responsiveness. From left to right, the first graph shows our control flow cytometry data with cells and no added agent. Within our quadrants of interest (Q2 and Q3), we found 1.81% of the cells. The next graph shows the cell data as responsive to pCD, where we found 3.44% of the cells from this sample in the same quadrants. The third graph was our target sample of interest with the treatment of pCD-Hyp as the incubating agent. 8.98% of cells were found to display signals in the green channel. The final graph is the sample treated with pyocyanin that is being

used as a positive control for the generation of reactive oxygen species (ROS). In this sample, 10.79% of cells were in quadrants 2 and 3. Figure 5d was set up in an identical condition to figure 5c, except that we were measuring hypoxia responsiveness. Therefore, the positive control was the hypoxia inducer deferoxamine. Within the regions of interest, the graphs had cell counts of 2.71%, 6%, 8.86% and 11.38% respectively. Similar experiments were also performed with MDA-MB-231 cells (Figure S5) which proved the lower ROS generation and hypoxia inside MDA-MB-231 cells compared to MCF-7.

As shown in figure 6a, we performed a series of experiments using hydrogen peroxide and lipase, both derived from human pancreas, to test whether our probe can safely break down in the human body. Figure 6b shows diminishing signals from our probe incubated with human lipase and hydrogen peroxide for increasing period of time. Figure 6c exhibits the absorbance profile of these samples where longer incubation time generally correlated with decreasing absorbance. The decrease in absorbance at 565 nm with increase in incubation period has been diagrammatically shown in figure 6d. Figure S6 represents the mass spectrometry data from pCD-Hyp alone and pCD-Hyp incubated with lipase for 48 hours showing that the larger peaks from pCD-Hyp disappeared and several smaller peaks showed up in the incubated sample indicating the degradation products.

Discussion

We found that pCD-Hyp has a fluorescence emission profile exceeding 700 nm in our proof-of-concept experiments. Consequently, this wavelength constitutes the minimum NIR window for in-vivo imaging. Using gel dock experiments to optimize NIDz concentration, the optimal

concentration between 50 and 100 mM was difficult to determine. Therefore, we were prompted to perform the quantitative ninhydrin assay experiments. Through this procedure, the optimal concentration was found to be 50 mM NIDz because the greatest surface coverage of pCD by NIDz was recorded at this concentration. At greater concentrations of NIDz, the mixture becomes oversaturated. The mechanism behind the increase in NIR emission for pCD-Hyp under hypoxic condition has been well validated by the reduction of nitro group present on NIDz to amine while incubating the pCD-Norm samples with human hemoglobin.

Using IVIS, we optimized our parameters with the highest excitation and emission wavelengths where we could still detect signals from our probe, which are 675 and 720 nm, respectively. Smaller excitation wavelengths produced stronger signals, but they would also excite biological molecules such as blood, which may interfere with our probe's ability to detect signals. In order to be well within the NIR range, 720 nm was chosen as the emission wavelength. To test pCD-Hyp's effectiveness in biological tissue free of tumors, we used pCD-Hyp to validate our hypothesis. In figure 4e, as the time point of imaging gradually increased from 0.5h to 2h, the radiant efficiency of the probe’s fluorescent signals began to level off or even increase in the 5 mm case. As no active vasculature is present to supply oxygen to the ex vivo tissue, the tissue is likely lacking oxygen. An important result is acquired from figures 4f and 4g where promising fluorescent signals were acquired from our probe when injected in the *ex vivo* tissue 5 mm deep. Our next step with IVIS would be to perform *in vivo* studies where we inject our probe intravenously into mice containing cancerous tumors.

The EPR measurements allowed us to differentiate nitroxide radicals from pCD-Hyp by distinguishing nitroxide radical responses [47]. Our probe was designed to measure both cell responses to oxidative stress and hypoxia since this radical induces oxidative stress. We performed

a MTT assay to quantify the damaging oxidative stress effect that the probe imposes on the cells. MCF-7 cells appeared to be more heavily affected by the oxidative stress generation than MDA-MB-231. We proceeded with flow cytometry using these cells to measure how the cells will be affected by our probe. With regard to the experiments to test for oxidative stress responses in cells, the percentage of cells that showed signals in the green channel from pCD-Hyp was much closer to our positive control than the percentage of cells that displayed signals from the detection reagent and pCD treatment. In fact, the presence of oxidative stress could dissipate after longer periods than the optimum time. From our hypoxia responsive cell data, we noticed a slight increase in the percentage of cells found in the red signal with pCD-Hyp compared to our controls, although noticeably less than the difference we recorded from the oxidative stress experiments. This may be due to a difference in the time of our probe's induction of hypoxia on the cells compared to the positive control's induction of hypoxia on the cells. In general, our probe successfully displayed hypoxia-responsiveness and the production of reactive oxygen species that induce oxidative stress in cells. Oxidative stress was important to measure as it could potentially be used in photothermal therapies to treat cancer in future work.

The biodegradability experiments provided us with some promising data as well. The absorbance in the region where we initially noticed an increase from pCD-Hyp was virtually nonexistent after 48 hours of treatment with the lipase enzyme. Using gel dock luminescence imaging at various time points, the probe fully degraded within 48 hours. Following 48 hours of lipase treatment, the larger peaks found in the mass spectrometry data have disappeared, indicating that the most abundant compounds in pCD-Hyp have disappeared. Smaller peaks identify the compounds that break down from large compounds. Even though these peaks have not yet been

characterized to determine the precise compounds being broken and resolved, degradation of the probe in the presence of the enzyme is evident.

Summary points.

- The mechanism behind the increase in NIR emission for pCD-Hyp under hypoxic condition has been well validated by the reduction of nitro group present on NIDz to amine.
- This probe exhibits hypoxia-sensitive fluorescence emission (600-820nm) has a deep biological tissue range for fluorescence imaging, and generates oxidative stress, which may be useful for future cancer therapies, such as photothermal therapy.
- Promising fluorescent signals were acquired from our probe when injected in the *ex vivo* tissue 5 mm deep.
- The EPR measurements allowed us to differentiate nitroxide radicals from pCD-Hyp by distinguishing nitroxide radical responses.
- Our probe was designed to measure both cell responses to oxidative stress and hypoxia since this radical induces oxidative stress.
- We performed a MTT assay to quantify the damaging oxidative stress effect that the probe imposes on the cells.
- In general, our probe successfully displayed hypoxia-responsiveness and the production of reactive oxygen species that induce oxidative stress in cells.

Conclusion.

Researchers have studied hypoxia in a variety of fields, including developmental biology and embryology. In addition to cancer, hypoxia also plays a pathophysiological role in myocardial ischemia, metabolic diseases, chronic kidney disease, and reproductive disorders. Solid tumors suffer from nutrient and oxygen deprivation due to rapid proliferation of cancer cells. Cancer cells can adapt to hypoxic conditions and persist to promote cancer progression. The prognosis of patients with intratumoral hypoxia has consistently been worse. Considering the potential clinical importance of hypoxia in cancer progression, it is increasingly critical to develop methods for measuring O_2 concentrations. Direct measurement of O_2 levels is possible using needle-type probes, both optically and electrochemically. Additionally, immunolabeling endogenous or exogenous markers has been optimized as an indirect, noninvasive approach. The use of fluorescent, phosphorescent, and luminescent reporters has also been used experimentally to measure O_2 in tumors or live cells. Experimentally detecting and monitoring hypoxic O_2 levels remains challenging, despite significant efforts. It may be difficult to adapt some of the described methods or specialized equipment may be required to measure actual levels of oxygen. We have developed here a relatively simple carbon dot-based probe that exhibits reversible fluorescence switching behaviors under normoxic and hypoxic conditions. This probe exhibits hypoxia-sensitive fluorescence emission (600-820nm) has a deep biological tissue range for fluorescence imaging, and generates oxidative stress, which may be useful for future cancer therapies, such as photothermal therapy. Additionally, it was demonstrated to be able to degrade successfully in human lipase presence and to exhibit versatility by displaying iron reactivity. We see this as a promising and useful development for early cancer diagnostics coupled with in vivo biological imaging.

AUTHOR INFORMATION

Corresponding Author

*dipanjan@psu.edu

CRediT authorship contribution statement

Conceptualization (IS, DP); Investigation (IS, KB, KW, EA, SP), Visualization (IS, KB, PM); Supervision (DP); Writing – original draft (IS, KB); Writing – review & editing (PM, DP).

Notes

Prof. Pan is the founder or co-founder of three University based start-ups. None of these entities, however, supported this work. All the other authors have no conflicts to declare.

ACKNOWLEDGMENT

We thank the Frederick Seitz Materials Research lab, Roger Adams lab, Noyes lab, and Beckman Institute for analytical experiments. We thank Dr. Toby Woods for performing the EPR spectroscopy experiments. Authors gratefully acknowledge the National Science Foundation for partially supporting this work (Award Number 2229986).

Figure legends

Figure 1. Schematic representation of the synthesis and reduction mechanism of the hypoxic probe. The carbon dot probe is solvothermally synthesized from p-phenylenediamine and subsequently functionalized with a small molecule, NIDz. Under hypoxic condition, the nitro

group of the probe sequentially reduces to an amine, generating NIR emission. The probe is also found to be reversible and does not emit under normoxic conditions.

Figure 2. Physico-chemical characterization and optimization experiments of CDs. (a) TEM image of p-CDs. (b) Electron paramagnetic resonance (EPR) spectrum of pCD, pCD-Norm, and pCD-Hyp. (c) UV-Visible spectroscopy of pCD without functionalization, pCD-Norm and pCD-Hyp. (d) Fluorescence spectroscopy of pCD without functionalization, pCD-Norm and pCD-Hyp. (e) Gel dock luminescence imaging of pCD samples with different concentrations of NIDz at normoxic and hypoxic environment. From 1 to 8, the NIDz concentrations are 0, 1.25, 2.50, 5.00, 10.0, 20.0, 50.0, and 100 mM respectively added to the pCD samples. (f) Percent coverage of pCD with NIDz in normoxia obtained from ninhydrin assay.

Figure 3. The reduction process of pCD-Norm in presence of haemoglobin has been schematically represented on top. The increase in (a) absorbance and (b) fluorescence emission of pCD-Norm with increase in concentration of haemoglobin has been demonstrated.

Figure 4. IVIS experiments with *ex vivo* tissue. (a) Schematic representation of probe preparation and injection into tissue for imaging. **(b)** IVIS images of pCD-Norm and pCD-Hyp at emission wavelength of 720nm when excited at wavelengths (570nm-675nm). **(c)** Comparative radiant efficiency of pCD-Norm and pCD-Hyp at different excitation wavelengths. **(d)** Fluorescent images from *ex vivo* chicken breast tissue with pCD-Hyp injected at 2.5 and 5 mm deep. **(e)** ROI measurements displaying the fluorescent signals over time following probe injection. **(f)** Fluorescent images at different probe injection depths: top to bottom is with increasing tissue depth. **(g)** Corresponding ROI measurements demonstrating the axial range the probe.

Figure 5. In vitro cell experiments with the probe. MTT assay results displaying cell viability of **(a)** MCF-7 and **(b)** MDA-MB-231 cells with our probe. **(c)** Oxidative Stress samples after 0.5 hours of MCF-7 cells incubated with detection mix alone, pCD, pCD-Hyp and an oxidative stress inducer positive control. **(d)** Hypoxia samples after 3.5 hours of MCF-7 cells incubated with detection mix alone, pCD, pCD-Hyp and a hypoxia inducer positive control.

Figure 6. Degradability experiments with human lipase. (a) Schematic representation of pCD-Hyp degradation process with human lipase and hydrogen peroxide. **(b)** Gel dock luminescence image of pCD-Hyp after increasing lengths of incubation with human lipase. **(c)** UV-Vis spectroscopy of pCD-Hyp after increasing lengths of incubation with human lipase. **(d)** Change in absorbance of pCD-Hyp at 565 nm with increasing lipase incubation.

Figure S1. Hydrodynamic diameter of pCDs as obtained *via* dynamic light scattering experiments.

Figure S2. Atomic force microscopy image of p-CDs: (a) topography image and (b) height profile.

Figure S3. Electron paramagnetic resonance (EPR) spectrum of (a) pCD, (b) pCD-Norm, and (c) pCD-Hyp.

Figure S4. Quantitative assessment of degree of hypoxia using the CDots-based probe in normoxic environment (pCD-Norm) and hypoxic environment (pCD-Hyp).

Figure S5. (a) Oxidative Stress samples after 0.5 hours of MDA-MB231 cells incubation with detection mix alone, pCD, pCD-Hyp and with an oxidative stress inducer positive control. **(b)** Hypoxia samples after 3.5 hours of MDA-MB231 cells incubated with detection mix alone, pCD, pCD-Hyp and with a hypoxia inducer positive control.

Figure S6. Mass spectrometry data of pCD-Hyp alone and pCD-Hyp after 48 hours incubation with human lipase.

References.

1. Xu C, Zou H, Zhao Z, et al. A New Strategy toward "Simple" Water-Soluble AIE Probes for Hypoxia Detection, *Adv. Func. Mater.* 29, 1903278 (2019).
2. Vaupel P, Harrison L, Tumor hypoxia: causative factors, compensatory mechanisms, and cellular response. *The Oncologist* 9(Supplement 5), 4-9 (2004). (*) - Understanding hypoxia
3. Cao Y, Li CY, Moeller BJ, et. al. Observation of incipient tumor angiogenesis that is independent of hypoxia and hypoxia inducible factor-1 activation. *Cancer Res.* 65(13), 5498-5505 (2005).
4. Takahashi S, Piao W, Matsumura Y, et. al. Reversible off–on fluorescence probe for hypoxia and imaging of hypoxia–normoxia cycles in live cells. *J. Am. Chem. Soc.* 134(48), 19588-19591 (2012). (**) - Probe differentiating hypoxic vs. normoxic conditions
5. Knox HJ, Hedhli J, Kim T, et al. A bioreducible N-oxide-based probe for photoacoustic imaging of hypoxia. *Nat. Commun.* 8(1), 1794 (2017).
6. Zhang M, Zhou Z, Zhao Z, Garrison J, Targeting Tumor Hypoxia in Radiotherapy: A Brief Review of Historical Background and Recent Progress. *Exploratory Res. Hyp. Med.* 1(1), 17-28 (2016).
7. Gross MW, Karbach U, Groebe K, et al. Calibration of misonidazole labeling by simultaneous measurement of oxygen tension and labeling density in multicellular spheroids. *Int. J. Cancer* 61(4), 567-573 (1995).

8. Misra SK, Srivastava I, Tripathi I, et al. Macromolecularly “caged” carbon nanoparticles for intracellular trafficking via switchable photoluminescence. *J. Am. Chem. Soc.* 139(5), 1746-1749 (2017).
9. Zhao X-B, Kang J-Y, Shi Y-P, Noncovalent dual-locked near-infrared fluorescent probe for precise imaging of tumor via hypoxia/glutathione activation. *Anal. Chem.* 94(17), 6574-6581 (2022). (**) - Precise imaging of tumor via hypoxia/glutathione activation
10. Zhang X, Zhang L, Wang X, et al. Visualizing and evaluating mitochondrial cysteine via near-infrared fluorescence imaging in cells, tissues and in vivo under hypoxia/reperfusion stress. *J. Hazardous Mater.* 419, 126476 (2021).
11. Atkinson KM, Morsby JJ, Kommidi SSR, et al. Generalizable synthesis of bioresponsive near-infrared fluorescent probes: sulfonated heptamethine cyanine prototype for imaging cell hypoxia. *Org. Biomol. Chem.* 19, 4100-4106 (2021).
12. Wang Y, Chen L, Smart fluorescent probe strategy for precision targeting hypoxic tumor. *J. Med. Chem.* 64(6), 2967-2970 (2021). (*) - Fluorescent probe strategy for precision targeting
13. Zhang X, Zhang L, Gao M, et al. A near-infrared fluorescent probe for observing thionitrous acid-mediated hydrogen polysulfides formation and fluctuation in cells and in vivo under hypoxia stress. *J. Hazardous Mater.* 396, 122673 (2020).
14. Wang H-S, Zhang X-F, Dong H, et al. Activatable near-infrared fluorescent probe triggered by nitroreductase for in vivo ulcerative colitis hypoxia imaging. *Anal. Chim. Acta.* 1221, 340107 (2022).
15. Zhou F, Zanganeh S, Mohammad I, et al. Targeting tumor hypoxia: a third generation 2-nitroimidazole-indocyanine dye-conjugate with improved fluorescent yield. *Org. Biomol. Chem.* 13(46), 11220-11227 (2015). (*) - Targeting tumor hypoxia with a third-generation dye-

conjugate with improved fluorescent yield

16. Xu Y, Zanganeh S, Mohammad I, et al. Targeting tumor hypoxia with 2-nitroimidazole-indocyanine green dye conjugates. *J. Biomed. Optics* 18(6), 066009 (2013).
17. Apte S, Chin F, Graves E, Molecular imaging of hypoxia: strategies for probe design and application. *Curr. Org. Synth.* 8(4), 593-603 (2011).
18. Smith AM, Mancini MC, Nie S, Bioimaging: second window for in vivo imaging. *Nat. Nanotech.* 4(11), 710 (2009).
19. Faber DJ, Aalders MC, Mik EG, et al. Oxygen saturation-dependent absorption and scattering of blood. *Phys. Rev. Lett.* 93(2), 028102 (2004).
20. Yang D, Tian HY, Zang T, et al. Hypoxia imaging in cells and tumor tissues using a highly selective fluorescent nitroreductase probe. *Sci. Rep.* 7(1), 9174 (2017).
21. Sun SW, Lin YC, Weng YM, Chen MJ, Efficiency improvements on ninhydrin method for amino acid quantification. *J. Food Compost. Anal.* 19(2-3), 112-117 (2006). (*) - Ninhydrin method for amino acid quantification
22. Suzuki T, Miyazaki C, Ishii-Watabe A, et al. A fluorescent imaging method for analyzing the biodistribution of therapeutic monoclonal antibodies that can distinguish intact antibodies from their breakdown products. *MAbs* 7(4), 759-769 (2015). (*) - Fluorescent imaging method for analyzing the biodistribution of therapeutic antibodies
23. Vitzthum F, Geiger G, Bisswanger H, et al. A quantitative fluorescence-based microplate assay for the determination of double-stranded DNA using SYBR Green I and a standard ultraviolet transilluminator gel imaging system. *Anal. Biochem.* 276(1), 59-64 (1999). (*) - Quantitative fluorescence-based microplate assay for the determination of double-stranded DNA
24. Abragam A, Bleaney B, *Electron paramagnetic resonance of transition ions*. OUP Oxford,

(2012).

25. Patterson WR, Poulos TL, Crystal structure of recombinant pea cytosolic ascorbate peroxidase. *Biochemistry* 34, 4331-4341 (1995).
26. Mukherjee P, Misra SK, Gryka MC, Chang HH, Tiwari S, Wilson WL, Scott JW, Bhargava R, Pan D, Tunable Luminescent Carbon Nanospheres with Well-Defined Nanoscale Chemistry for Synchronized Imaging and Therapy. *Small* 11, 4691–4703 (2015).
27. Pham CTN, Thomas DG, Beiser J, Mitchell LM, Huang JL, Senpan A, Hu G, Gordon M, Baker NA, Pan D, Lanza GM, Hourcade DE, Application of a hemolysis assay for analysis of complement activation by perfluorocarbon nanoparticles. *Nanomed. Nanotech. Biol. Med.* 10, 651–660 (2014).
28. Ostadhossein F, Pan D, Functional carbon nanodots for multiscale imaging and therapy. *WIREs Nanomed. Nanobiotechnol.* 9, e1436 (2017).
29. Srivastava I, Misra SK, Ostadhossein F, Daza E, Singh J, Pan D, Surface chemistry of carbon nanoparticles functionally select their uptake in various stages of cancer cells. *Nano Res.* 10, 3269–3284 (2017).
30. Misra SK, Chang HH, Mukherjee P, Tiwari S, Ohoka A, Pan D, Regulating Biocompatibility of Carbon Spheres via Defined Nanoscale Chemistry and a Careful Selection of Surface Functionalities. *Sci. Rep.* 5, 14986 (2015).
31. Misra SK, Ostadhossein F, Daza E, Johnson EV, Pan D, Hyperspectral Imaging Offers Visual and Quantitative Evidence of Drug Release from Zwitterionic-Phospholipid-Nanocarbon When Concurrently Tracked in 3D Intracellular Space. *Adv. Func. Mater.* 26, 8031–8041 (2016).
32. Srivastava I, Khamo JS, Pandit S, et al. Influence of Electron Acceptor and Electron Donor on

the Photophysical Properties of Carbon Dots: A Comparative Investigation at the Bulk-State and Single-Particle Level. *Adv. Func. Mater.* 29, 1902466 (2019).

33. Misra SK, Ohoka A, Kolmodin NJ, Pan D, Next generation carbon nanoparticles for efficient gene therapy. *Mol. Pharmaceut.* 12, 375–385 (2015).
34. Pan D, Pramanik M, Wickline SA, Wang LV, Lanza GM, Recent advances in colloidal gold nanobeacons for molecular photoacoustic imaging. *Contrast Media Mol. Imaging* 6, 378–388 (2011).
35. Pan D, Pramanik M, Senpan A, Wickline SA, Wang LV, Lanza GM, A facile synthesis of novel self-assembled gold nanorods designed for near-infrared imaging. *J. Nanosci. Nanotechnol.* 10, 8118–8123 (2010).
36. Khan MS, Misra SK, Dighe K, Wang Z, Schwartz-Duval AS, Sar D, Pan D, Electrically-receptive and thermally-responsive paper-based sensor chip for rapid detection of bacterial cells. *Biosens. Bioelectron.* 110, 132–140 (2018).
37. Pan D, Schmieder AH, Wang K, et al. Anti-Angiogenesis Therapy in the Vx2 Rabbit Cancer Model with a Lipase-cleavable Sn 2 Taxane Phospholipid Prodrug using αvβ3-Targeted Theranostic Nanoparticles. *Theranostics* 4, 565–578 (2014).
38. Moitra P, Alafeef M, Dighe K, Sheffield Z, Dahal D, Pan D, Synthesis and Characterization of N-gene targeted NIR-II Fluorescent Probe for Selective Localisation of SARS-CoV-2. *Chem. Commun.* 57, 6229-6232 (2021).
39. Moitra P, Chaichi A, Hasan SMA, et al. Probing the mutation independent interaction of DNA probes with SARS-CoV-2 variants through a combination of surface-enhanced Raman scattering and machine learning. *Biosens. Bioelectron.* 208, 114200 (2022).
40. Misra SK, Jensen TW, Pan D, Enriched inhibition of cancer and stem-like cancer cells via

STAT-3 modulating niclocelles. *Nanoscale* 7, 7127–7132 (2015).

41. Becker ML, Remsen EE, Pan D, Wooley KL, Peptide-derivatized shell-cross-linked nanoparticles. 1. Synthesis and characterization. *Bioconjugate Chem.* 15, 699–709 (2004).
42. Moitra P, Alafeef M, Dighe K, Pan D, Single-gene diagnostic assay for rapid subclassification of basal like breast cancer with mRNA targeted antisense oligonucleotide capped molecular probe. *Biosens. Bioelectron.* 207, 114178 (2022).
43. Ostadhossein F, Moitra P, Gunaseelan N, et al. Hitchhiking probiotic vectors to deliver ultra-small hafnia nanoparticles for 'Color' gastrointestinal tract photon counting X-ray imaging. *Nanoscale Horiz.* 7, 533-542 (2022).
44. Moitra P, Iftesum M, Skrodzki D, et al. Nucleotide-Driven Molecular Sensing of Monkeypox Virus Through Hierarchical Self-Assembly of 2D Hafnium Disulfide Nanoplatelets and Gold Nanospheres. *Adv. Func. Mater.* 33, 2212569 (2023).
45. Pradhan J, Moitra P, Umesh, et al. Encapsulation of CsPbBr3Nanocrystals by a Tripodal Amine Markedly Improves Photoluminescence and Stability Concomitantly via Anion Defect Elimination. *Chem. Mater.* 32, 7159-7171 (2020).
46. Misra SK, Moitra P, Kondaiah P, Bhattacharya S, Co-liposomes having anisamide tagged lipid and cholesteryl tryptophan trigger enhanced gene transfection in sigma receptor positive cells. *Colloid Surf. B: Biointerfaces* 142, 130-140 (2016).
47. Srivastava I, Lew B, Wang Y, et al. Cell-membrane coated nanoparticles for tumor delineation and qualitative estimation of cancer biomarkers at single wavelength excitation in murine and phantom models. *ACS Nano* 17 (9), 8465-8482 (2023).