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Hybrid TiO_2 -ruthenium nano-photosensitizer synergistically produces reactive oxygen species in both hypoxic and normoxic conditions

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Abstract

Photodynamic therapy (PDT) is widely used to treat diverse diseases, but its dependence on oxygen to produce cytotoxic reactive oxygen species (ROS) diminishes the therapeutic effect in a hypoxic environment such as solid tumors. Here, we developed a ROS-producing hybrid nanoparticle-based photosensitizer capable of maintaining high levels of ROS under both normoxic and hypoxic conditions. Conjugation of an organometallic ruthenium complex (N3) to a TiO_2 nanoparticle afforded TiO_2 -N3. Upon exposure of TiO_2 -N3 to light, the N3 injected electrons into TiO_2 to produce three- and four-fold greater hydroxyl radicals and hydrogen peroxide, respectively, than TiO_2 at 160 mmHg. TiO_2 -N3 maintained three-fold higher hydroxyl radicals than TiO_2 under hypoxic conditions via N3-facilitated electron hole reduction of adsorbed water molecules. The incorporation of N3 transformed TiO_2 from a dual type I and II PDT agent to a predominantly type I photosensitizer, irrespective of the oxygen content.

COMMUNICATION

A dual type I and type II PDT agent, titanium dioxide-ruthenium nano-photosensitizer (TiO_2 -N3) synergistically produced hydroxyl radicals under both hypoxic and normoxic conditions.

Keywords

nanophotosensitizer; TiO₂; reactive oxygen species; hypoxia; organometallic ruthenium complex

Reactive oxygen species (ROS) are products of normal metabolic processes in living organisms. At low concentrations, these species play vital roles in cell signaling, but they can become cytotoxic at elevated levels via reaction with proteins, DNAs, lipids, and other biological molecules. Photodynamic therapy (PDT) is a treatment paradigm that selectively elevates ROS levels to induce cell death by multiple pathways.^[1] Some molecules, known as photosensitizers (PSs), absorb light of the appropriate wavelengths to produce cytotoxic free radicals and ROS. Many organic PSs mainly produce singlet oxygen (¹O₂) formed via spin inversion of triplet oxygen to singlet oxygen (type II PDT).^[2] Specific organic PSs can also produce hydrogen peroxide (H₂O₂) and hydroxyl ([•]OH) radicals, formed from direct ionization events (type I PDT).^[2] While the highly reactive [•]OH and ¹O₂ rapidly interact with adjacent biomolecules to disrupt normal cell functions and induce cell death, the more stable H₂O₂ can travel across cell membranes.^[3] Intracellular scavengers, such as catalases or peroxidases can convert H₂O₂ to superoxide radicals.^[4] However, ferrous ions can rapidly oxidize H₂O₂ to reactive [•]OH radicals (Fenton reaction) to enhance the PDT effect, especially in tumor cells where the increased demand for iron is high.^[5] Despite these advantages, the reliance of many organic PSs on molecular oxygen limits the effectiveness of PDT in hypoxic environments, which include many solid tumors.^[6] During PDT, local oxygen is rapidly consumed, creating transient hypoxia that further decreases the therapeutic effects.^[7] Moreover, most organic PSs can react with ¹O₂ produced during PDT, leading to a depletion of the PSs' concentration via a photobleaching process.^[8] As a result, a recent trend in PDT research includes the development of new PSs that are capable of producing cytotoxic ROS, irrespective of the oxygenation status of the microenvironment.

Some studies have shown that organometallic PSs can damage DNA under normoxic and hypoxic conditions.^[9] One of such complexes is *cis*-bis(isothiocyanato)bis(2,2'-bipyridyl-4,4'-dicarboxylato)ruthenium(II), abbreviated N3. This complex, which absorbs light in the UV and visible wavelengths, possesses multiple triplet states that can be populated by both metal to ligand charge transfer (³MLCT) and intra-ligand charge transfer (³π-π*) mechanisms.^[10] Organometallic PSs have long triplet state lifetimes (~ms) that enhances the interaction with oxygen species and subsequent ROS production.^[11] Although N3 can produce damage regardless of molecular oxygen content, metal oxides such as TiO₂ produce higher amounts of ROS in the presence of oxygen. The excitation of TiO₂ with radiative energy greater than its band gap of 3.2 eV (387 nm) promotes an electron to the conduction band, creating an electron hole pair.^[12] The electrons primarily reduce adsorbed molecular oxygen to superoxide, and the holes largely oxidize substrates such as water into a hydrogen ion and an [•]OH.^[12] These processes allow TiO₂ to produce higher amounts of diverse ROS compared to traditional small molecule PS, making TiO₂ a viable PDT agent. However, hypoxic conditions significantly deplete the amount of ROS that TiO₂ forms.

In this study, we synthesized TiO₂-N3 nanoparticles, consisting of a regenerative ROS-producing TiO₂ core that is coated with N3 to enhance ROS production at different levels of

oxygen pressure. TiO₂ materials are excellent electron acceptors from organometallic ruthenium complexes, a phenomenon that has been used for converting sunlight into current.^[13] By coating the surface of TiO₂ nanoparticles with N3, each ruthenium complex can inject an electron into TiO₂, building up electrons that increase ROS production.^[12, 13b, 14] TiO₂ is widely considered biologically inert and toxicity studies conducted in tumor bearing mice demonstrate limited dark toxicity.^[13e] Similarly, toxicity studies with organometallic ruthenium complexes have also been conducted in tumor cells and mice, and show limited toxicity.^[9b]

TiO₂-N3 nanoparticles were prepared by shaking a mixture of 25 nm sized anatase TiO₂ particles and N3 in an ethanol overnight. The particles were then centrifuged and the supernatant discarded, followed by the addition of water to the residue. Subsequent sonication (45 seconds) dispersed and evenly coat the particles. Filtration through a 0.45 μ m pore removed large aggregates. The absorption spectra of N3 blue-shifts when coated on TiO₂, suggesting that TiO₂ increased the excited state energies of the N3 (Figure 1a). The TiO₂-N3 particles contain an average of 125,000 N3 molecules per nanoparticle (Figure 1b) with an average hydrodynamic size of 135 nm based on dynamic light scattering measurement (Figure 1c). Transmission electron microscopy of TiO₂-N3 showed that the 25 nm TiO₂ cores were maintained in the 135 nm clusters (Figure 1d).

To determine the ROS produced by TiO₂-N3, bare TiO₂, and N3, we used hydroxyphenyl fluorescein (HPF), singlet oxygen sensor green (SOSG), and Amplex Red assay for $\cdot$ OH, ¹O₂, and H₂O₂, respectively. For these assays TiO₂-N3 was not sonicated or filtered. A solution of 10 μ M HPF or SOSG and PS (0.1 mg/mL TiO₂, 0.1 mg/mL TiO₂-N3, or 7 μ M N3) was irradiated at 1.69 mW/cm² using a 365 nm light source. For $\cdot$ OH, measurements, aliquots of 100 μ L of HPF were diluted in Tris buffer (pH 8.0; 900 μ L) at different time points (Figure 2a) and the fluorescence of each sample was measured at ex/em 478/515 nm. ¹O₂ measurements were accomplished by irradiating SOSG in a quartz cuvette continuously for 60 min and the fluorescence was measured every 1 min (ex/em 504/525 nm). H₂O₂ measurements were performed in a 96 well plate (50 μ L) using the same concentrations of each PS as above and an irradiation power of 2.39 mW/cm². The fluorescence at ex/em 545/590 nm and a reference standard were used to calculate the concentration of H₂O₂ in each well. To quantify the amount of the different types of ROS produced, each activation curve was fitted to an exponential plot (Figure 2a–c).

Scheme 1 illustrates the different pathways to generate ROS ($\cdot$ OH, H₂O₂, and ¹O₂) from TiO₂-N3. Under normal oxygen pressure (160 mmHg), the photo-activation of TiO₂-N3 resulted in about 3-fold higher $\cdot$ OH production compared to TiO₂ and N3 (Figure 2a). These radicals could be produced by two mechanisms. Reaction 1 is a one-step oxidation of water to a $\cdot$ OH, a primary pathway used by bare TiO₂ nanoparticles (Scheme 1a). The observed increase in $\cdot$ OH suggested the involvement of another mechanism, Reaction 2, which reduces a precursor H₂O₂ to an $\cdot$ OH. Two pathways are available for H₂O₂ formation (Scheme 1b). In Reaction 3, two conduction band electrons from TiO₂ are used to first reduce oxygen to superoxide, which is subsequently reduced to H₂O₂. In the second mechanism, Reaction 4, two holes on the TiO₂ nanoparticles oxidize adsorbed water molecules to H₂O₂. The complementary molecular oxygen dependent (Reaction 3) and

independent (Reaction 4) pathways to produce H_2O_2 ensures the generation of hydroxyl radicals. The bare TiO_2 particles probably rely more on Reaction 1 than 2 for $\cdot\text{OH}$ production because of the direct oxidation of abundant water molecules. Alternatively, the TiO_2 in $\text{TiO}_2\text{-N}_3$ has higher numbers of conduction band elections due to the injection of electrons from photo-activated N_3 . This process creates more electrons for Reactions 3 and 2, accounting for the observed 3-fold increase in the $\cdot\text{OH}$ produced (Scheme 1d).

We next postulated that the high $\cdot\text{OH}$ levels in $\text{TiO}_2\text{-N}_3$ are associated with a corresponding increase in H_2O_2 production. Upon photoactivation, $\text{TiO}_2\text{-N}_3$ generated about 4-fold higher H_2O_2 than TiO_2 (Figure 2b). The low yield of H_2O_2 from N_3 indicates that its contribution to $\cdot\text{OH}$ production was minimal. These results support a mechanism where N_3 injected electrons into TiO_2 to increase the conversion of oxygen to H_2O_2 via Reaction 3 and a rapid transformation of H_2O_2 to $\cdot\text{OH}$ through Reaction 2.

The other reactive ROS, $^1\text{O}_2$, is the primary species involved in oxygen dependent type II PDT. The ease of electron-hole pair generation upon exposure of TiO_2 nanoparticles to light, facilitates concerted reduction of oxygen to super oxide and subsequent oxidation to $^1\text{O}_2$ (Scheme 1c). Expectedly, our results show that TiO_2 -generated $^1\text{O}_2$ was an order of magnitude higher than $\text{TiO}_2\text{-N}_3$ or N_3 (Figure 2c). In contrast to the synergistic effects of N_3 on $\cdot\text{OH}$ and H_2O_2 production by $\text{TiO}_2\text{-N}_3$, N_3 effectively inhibited $^1\text{O}_2$ generation. Alone, N_3 can produce singlet oxygen through intersystem crossing to the triplet state and spin-inversion of the triplet state of N_3 and triplet state of oxygen. Whereas this process takes place on the time scale of microseconds,^[11] electron injection into TiO_2 occurs on the time scale femtoseconds.^[15] As a result, when N_3 is adsorbed onto TiO_2 , the probability of electron injection is higher than intersystem crossing and spin-inversion. Thus, N_3 favors reduction reactions, which enhances H_2O_2 production that is subsequently used to increase $\cdot\text{OH}$. Effectively, N_3 converts TiO_2 from a dual type I and II PDT agent to a predominantly type I PS under normoxic conditions.

To test the performance of the PSs under severe hypoxic conditions, we bubbled nitrogen in a suspension of the PSs and the appropriate sensors to attain 8 mmHg, the average partial pressure of oxygen in hypoxic tumors, for all ROS measurements.^[16] For H_2O_2 and $\cdot\text{OH}$ measurements, the solution was then added to a 96-well plate and incubated in 8 mmHg oxygen pressure atmosphere for an hour. Measurements were taken at different time points after UV irradiation of the solution (Figure 3). We found that the $\cdot\text{OH}$ production followed the same trend as in normoxic conditions, with $\text{TiO}_2\text{-N}_3$ generating about 3-fold greater $\cdot\text{OH}$ than either TiO_2 or N_3 (Figure 3a). In addition, the rate of $\cdot\text{OH}$ production for $\text{TiO}_2\text{-N}_3$ was 3-fold higher than TiO_2 . These results demonstrate that the reductive effect of N_3 on TiO_2 allowed the efficient utilization of oxygen, even at low pressures, to maintain $\cdot\text{OH}$ production (Figure 3d).

N_3 was the only PS that generated H_2O_2 in hypoxic conditions (Figure 3b, e). The slopes of H_2O_2 generation for TiO_2 and $\text{TiO}_2\text{-N}_3$ were both negative in hypoxic conditions, indicating a decrease in baseline H_2O_2 over time. The dependence of the initial on the reduction of oxygen reaction to produce H_2O_2 led to the observed effect for TiO_2 or $\text{TiO}_2\text{-N}_3$ (Reaction

3). Irrespective of the PS used, the minimal availability of molecular oxygen under hypoxic conditions prevented the production of $^1\text{O}_2$ (Figure 3c, f).

This work demonstrates that combining the two PSs resulted in changes to their photophysical properties, which altered both the type and amount of ROS produced. It also delineates the various pathways that $\text{TiO}_2\text{-N3}$ uses to enhance $\bullet\text{OH}$ production and maintain a significant level of these species in normoxic and hypoxic conditions. The addition of N3 to TiO_2 causes a build-up of electrons on the TiO_2 surface, which likely accounts for the synergistic production of H_2O_2 and $\bullet\text{OH}$ (Figure 2 a, b and Scheme 1d). Furthermore, the inability of TiO_2 to produce H_2O_2 under hypoxic conditions suggests that N3 facilitated the depletion of residual H_2O_2 (Figure 3b, e) and the rapid conversion of the low oxygen concentration into $\bullet\text{OH}$, thus increasing the concentration of this species (Figure 3a, d). The ensemble of these results demonstrates a mechanism to convert and maintain cytotoxic $\bullet\text{OH}$ production by harnessing the reductive power of ruthenium complexes, which efficiently reduces low levels of oxygen for ROS production. The limited penetration of light in tissue precludes the routine use of these UV-absorbing materials for treating lesions deeper than a few microns from the light source. However, a recent work demonstrating the use of Cerenkov radiation from radionuclides as tissue-depth independent light source for PDT suggests the potential use of $\text{TiO}_2\text{-N3}$ nanoparticles for whole-body treatment of localized and disseminated disease.^[13e] Our findings could enable the application of this strategy to develop highly efficient nano-photosensitizers in the future for PDT.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Characterization of TiO₂-N3 nanoparticles. (a) Absorption spectra for N3 and TiO₂-N3. (b) Number of N3 incorporated into a 25 nm TiO₂ particle. (c) Average size of TiO₂-N3 particles after sonication and filtering. (d) Transmission electron microscopy of TiO₂-N3 particles with zeta potential of +11.2 ± 0.6 mV.

Figure 2. ROS production and characterization. Time course of (a) hydroxyl radical, (b) hydrogen peroxide, and (c) singlet oxygen production, respectively, as PSs are irradiated with 365 nm light.

Figure 3.

Oxygen dependence on hydroxyl radical production. ROS production of (a) hydroxyl radical, (b) hydrogen peroxide, and (c) singlet oxygen at 8 mmHg oxygen pressure. Rate of (d) hydroxyl radical, (e) hydrogen peroxide, and (f) singlet oxygen production under hypoxic conditions.

Scheme 1.

Schematic description of ROS generation by TiO_2 and electron injection to TiO_2 by N3, not drawn to scale.