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Review Article

Mechanistic characterization of titanium dioxide nanoparticle-induced toxicity using electron spin resonance☆

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ABSTRACT

Titanium dioxide nanoparticles (TiO_2 NPs) are one of the most widely used nanomaterials that have been manufactured worldwide and applied in different commercial realms. The well-recognized ability of TiO_2 to promote the formation of reactive oxygen species (ROS) has been extensively studied as one of the important mechanisms underlying TiO_2 NPs toxicity. As the "gold standard" method to quantify and identify ROS, electron spin resonance (ESR) spectroscopy has been employed in many studies aimed at evaluating TiO_2 NPs safety. This review aims to provide a thorough discussion of current studies using ESR as the primary method to unravel the mechanism of TiO_2 NPs toxicity. ESR spin label oximetry and immune-spin trapping techniques are also briefly introduced, because the combination of spin trapping/labeling techniques offers a promising tool for studying the oxidative damage caused by TiO_2 NPs.

1. Introduction

Titanium dioxide nanoparticles (TiO_2 NPs) have been widely applied as a coloring agent to provide whiteness and/or opacity in paints and personal care products, as well as being used as a food additive and a drug delivery agent. Moreover, due to their excellent UV absorbance and deflecting properties, TiO_2 NPs are a commonly used functional ingredient in cosmetics or skincare products to provide protection against sunlight. In environmental engineering, TiO_2 nanocomposites have been employed as a photocatalyst in water pollutant purification and hazardous chemical detoxification. When exposed to UV light, TiO_2 NPs absorb photons having an energy equal to or higher than its band gap (>3.0 eV), exciting electrons in the valence band to the conduction band. Photoexcitation, therefore, results in an increased number of conduction band electrons and consequently increased valence band holes. Electrons in the conduction band can reduce substrates in the chemical environment, for example, reduction of oxygen results in the formation of superoxide radical anions. Holes in the valence band can oxidize substrates such as water or hydroxide ions and generate hydroxyl radicals (•OH) [1,2]. Photocatalyzed chemical decomposition usually

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involves formation of reactive oxygen species (ROS), including superoxide radicals (O^{2-}•) and singlet oxygen ($^1O_2$) [3], as well as other intermediate species such as H_2O_2 or O_2 [1]. Because of those highly reactive free radicals generated during UV irradiation, engineered TiO_2 NPs have also been recognized for their light-induced biocidal effects against a broad range of harmful microorganisms, including bacteria such as *Escherichia coli* [4], molds such as *Aspergillus niger* [5], as well as protozoa such as *Giardia* and *Acanthamoeba* species [6].

Exposure to ROS derived from photoexcited TiO_2 NPs has raised concerns because ROS are believed to play an important role in many inflammatory skin disorders, skin aging, and cancer formation [7]. Due to the ability of nano-TiO_2 to induce ROS generation when irradiated, tremendous efforts have been focused on investigating potential risks associated with human exposure to TiO_2 NPs. In addition to direct exposure through consumption of products containing TiO_2 NPs, inhalation of NPs in the workplace, or through other environmental sources are possible exposure routes (e.g., emitted nanomaterials that reach the land can potentially contaminate soil and migrate to water systems) [8]. To date, various nanomaterial studies have linked toxicity to the production of ROS. It is well known that the generation of intercellular ROS can lead to oxidative stress, resulting in inflammation, immune response, cellular damage, and genotoxicity [9].

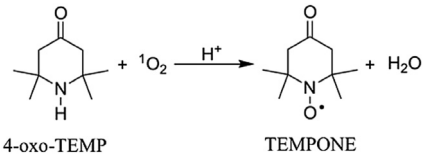
Free radicals, including ROS, are very short-lived entities, making them very difficult to detect when evaluating toxicity associated with oxidative stress. Electron spin resonance (ESR, also known as EPR, electron paramagnetic resonance) has been recognized as a "gold standard" and state-of-the-art tool for detecting and quantifying ROS in chemical and biological systems. Another ESR technique, ESR oximetry, has been used to monitor lipid peroxidation induced by highly reactive radicals. This review summarizes the advantages and recent developments using ESR as a tool to unravel the mechanism of nano-TiO_2-induced cytotoxicity and phototoxicity. In addition, immuno-spin trapping, another methodology based on the spin trapping technique to detect protein or DNA radicals, is briefly introduced. The combination of immuno-spin trapping with ESR spin trapping and ESR oximetry can provide a deep insight into the mechanism of ROS generation triggered by nanomaterials, as well as the subsequent oxidative damage to proteins, DNA, and lipids.

2. Electron spin resonance

2.1. ESR spin trapping

ESR is a spectroscopic technique used to detect chemical species with unpaired electrons. ESR has been recognized as the least ambiguous method for characterizing free radicals. Due to its high sensitivity and the ability to identify the generation of radicals *in situ*, the ESR spin trapping technique is commonly employed in nanoscience research to evaluate both the ROS scavenging capability of nanomaterials with regard to their potential applications in health promotion and cancer chemotherapy [10] and to investigate toxicities related to ROS generation. ESR spectroscopy has also been used for the validation of results obtained using other methods. For instance, the data from ESR spectroscopy using different spin probes – 1-hydroxy-3-carboxypyrrolidine and 4-phosphonooxy-2,2,6,6-tetramethylpiperidine-*N*-hydroxyl – showed good agreement with the data from confocal fluorescence imaging using different dyes, including 2′,7′-dichlorodihydrofluorescein diacetate, MitoSOX, and MitoTracker red CM-H_2XRos [11]. Moreover, the development of nontoxic spin traps makes it possible for the detection of free radicals both *in vivo* [12] and *ex vivo* [13].

ROS are low-level and short-lived free radicals, which are difficult to determine in chemical and biological systems. Spin trapping agents are therefore employed to intercept the target free radical and to form a relatively stable and distinguishable spin adduct that can be quantified and identified by ESR spectroscopy [10]. Based on their characteristic structures, spin traps can be divided into two groups: nitroso and nitrone. Nitroso spin traps are less readily used in biological studies because of high reactivity of their C-nitroso group [14]. The most commonly used nitrone spin traps include 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), α-phenyl-*N*-tert-butylnitrone (PBN) α-(4-pyridyl-1-oxide)-*N*-tert-butylnitrone (POBN), and 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO). The spin label probes 2,2,6,6-tetramethyl-4-piperidone (TEMP) and 4-oxo-2,2,6,6-tetramethyl-2-piperidone (4-oxo-TEMP) have been employed to detect singlet oxygen [15]. The reaction of $^1O_2$ with 4-oxo-TEMP leads to the formation of a nitroxide radical 4-oxo-2,2,6,6-tetramethylpiperidine-*N*-oxyl (TEMPONE) that exhibits a stable triplet ESR spectrum, Equation (1):



4-oxo-TEMP TEMPONE

(1)

In comparison with other nitrone spin traps, DMPO is generally preferable because of its low redox activity and the ability to yield ESR spectra that are highly dependent on the radical species. However, a major drawback of DMPO is that the decomposition of DMPO/•OOH to DMPO/•OH makes it difficult to distinguish between the formation of •OOH and •OH [16]. By contrast, 5-methyl-1-pyrroline *N*-oxide (BMPO) provides an ideal solution to this problem because of the formation of a more stable BMPO/•OOH adduct that does not decompose to BMPO/•OH [16]. BMPO/•OH has an ESR spectrum similar to that of DMPO/•OH, exhibiting a characteristic set of four lines (1:2:2:1) [2]. Another method to further distinguish whether the signal is from DMPO/•OH or DMPO/O^{2-}• spin adduct is the effects of superoxide dismutase (SOD) [17] or mannitol on the ESR spectrum [18]. Because the former only scavenges O^{2-}•, whereas the latter only reacts with •OH, the predominant species (•OH or O^{2-}•) in the system can be determined by observing changes of the ESR spectra.

2.2. ESR spin label oximetry

As aforementioned, ESR spectroscopy detects molecules with unpaired electrons. Theoretically, the direct detection of