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# Long-term bioavailability of redox nanoparticles effectively reduces organ dysfunctions and death in whole-body irradiated mice

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ABSTRACT

Radioprotective agents have been developed to protect patients against the damaging and lethal effects of ionizing radiation. However, in addition to the intrinsic ability to target reactive oxygen species (ROS), the ability to retain a significant level of bioavailability is desirable in radioprotective agents because that would increase and prolong their radioprotective efficacy and improve its safety. Here, we report the development of a novel nanoparticle-based radioprotective agent with improved bioavailability, which suppressed the adverse effects typically associated with low-molecular-weight (LMW) antioxidants. We developed biocompatible and colloidally stable nanoparticles in which nitroxide radicals that were covalently conjugated (redox nanoparticles, $RNP^N$) effectively scavenged radiation-induced ROS with a characteristically prolonged bioavailability and tissue-residence time compared with that of conventional LMW antioxidants. The confinement of the nitroxide radicals in the $RNP^N$ core prevented its rapid metabolism and excretion out of the body. The nano-sized formulation prevented internalization of $RNP^N$ in healthy cells, thereby preserving the normal function of the redox reactions in the cell. This improved pharmacological performance dramatically reduced the radiation-induced organ dysfunctions and increased the survival time of the lethally irradiated mice when the nanoparticles were administered 3 −24 h before whole-body irradiation.



## 1. Introduction

The increasing use of radioactive materials for various applications such as radiotherapy, medicine, diagnosis, and nuclear power generation requires the development of new radioprotective agents. Radioprotectors are compounds that are administered before exposure to ionizing radiation to protect healthy normal cells and reduce the damaging effects including radiation-induced lethality [1,2]. A potent radioprotector could be used for numerous applications including clinical radiotherapy, space travel, radiation site clean-up, radiological terrorists and military attacks, nuclear accidents, and the everyday protection of radiation workers [3].

Currently, only two radioprotective compounds have been approved by the U.S. Food and Drug Administration (FDA) for radiotherapy [4]. The first drug is amifostine, a cytoprotective agent, developed under the name WR-2721 by the US Walter Reed Army Institute of Research to protect soldiers against radiation exposure from nuclear weapons during the Cold War. Decades after its development, its clinical use was approved in 1995 to reduce the renal effects of radiation exposure and subsequently for protection

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from xerostomia (dryness of the mouth) induced by postoperative radiotherapy for head and neck cancer [5]. The second drug, palifermin, gained FDA approval in 2004 as a recombinant protein that decreases the incidence and duration of severe oral mucositis in patients undergoing chemotherapy and radiotherapy [6]. However, the use of these compounds has been limited because of some serious adverse effects [5]. These limitations have led to the evaluation of several compounds (e.g., phytochemicals) for possible radioprotective effects [7–11]. However, most of these compounds have not been successful in obtaining approval for clinical use owing to their instability, rapid excretion, toxicity [12], and low therapeutic potential. The slow-moving development of radioprotective agents and urgent unmet demand for radioprotectants with high efficacy and low toxicity [13], have prompted researchers to investigate the possibility of reinventing amifostine using nanotechnology. The use of poly(lactide-*co*-glycolide) (PLGA)/amifostine (Ethyol®) and PLGA/WR-1065 (an active metabolite of amifostine) nanoparticles has increased its oral bioavailability and its effectiveness was observed following its administration 1 h before irradiation [14,15]. Furthermore, highly catalytic nanodots have been used, but their rapid secretion limits their application and they were only effective if administered 0.5 h before irradiation [13].

Alternative promising candidates are low-molecular-weight (LMW) nitroxide compounds. Originally used as biophysical probes, stable nitroxide free radicals have been identified as novel antioxidants. They act as reducing agents, which catalytically scavenge oxidants by both oxidation and reduction. Among the numerous types of nitroxide radicals, 4-amino-2,2,6,6-tetramethylpiperidine-1-oxyl (4-amino-TEMPO, otherwise known as tempamine or $NH_2$-TEMPO) was identified to have the highest radioprotective efficacy *in vitro* [16]. However, like most LMW compounds, its bioavailability is extremely poor, compromising its therapeutic efficacy and limiting its clinical use. The drug's ability to achieve and maintain an adequate level in the blood circulation and its bioavailability, primarily in target tissue sites, are very important because they determine its therapeutic efficacy [17]. Therefore, we developed novel $NH_2$-TEMPO-containing redox nanoparticles ($RNP^N$) that effectively scavenge radiation-induced reactive oxygen species (ROS) with a characteristic long-term bioavailability and prolonged tissue-residence time. To prevent leakage of the LMW $NH_2$-TEMPO from the nanoparticles, it was covalently conjugated to the hydrophobic segment of the amphiphilic block copolymer. The synthesized nitroxide radical-containing block copolymer spontaneously forms core-shell type polymeric micelles in aqueous media [18]. The confinement of the nitroxide radicals in the $RNP^N$ core enabled the maintenance of significant amounts in the blood circulation and critical target organs for radiation protection. Its gradual disintegration allowed the exposure of the nitroxide core, which readily scavenged the short-lived radiation-induced ROS. This improved pharmacological performance dramatically reduced the radiation-induced organ dysfunctions and increased the survival time of the lethally irradiated mice when the nanoparticles were administered 3–24 h before total-body irradiation. Another important characteristic of the $RNP^N$ is its extremely low toxicity. In contrast to conventional antioxidants that can be internalized into healthy cells, which destroy its normal redox reactions (e.g., electron transport chain), the size of $RNP^N$ prevents its cellular internalization [19]. Our findings show that the intrinsic ROS targeting activity of the radioprotector is as important as its bioavailability at the site of action, especially in scavenging the short-lived radiation-induced ROS. Taken together, these results indicate that improving the drug's pharmacokinetics (PK) significantly increase its therapeutic efficacy, which subsequently breaks the boundaries of its applications.

## 2. Materials and methods

### 2.1. Design, synthesis, and characterization of $RNP^N$

#### 2.1.1. Synthesis of poly(ethylene glycol) (PEG)-b-chain transfer agent (CTA)

At 110 °C, 50 g of poly(ethylene glycol) monomethyl ether (MeO-PEG-OH, MW = 5,000, Fluka, Germany) was stirred under vacuum for 2–12 h or until no water or moisture remained. It was then incubated exposed to an atmosphere of nitrogen gas ($N_2$) for 1 h at 65 °C with stirring for stabilization. $N_2$ was introduced just before adding tetrahydrofuran (THF, 200 mL) slowly to the dehydrated polymer. The hydroxyl groups were lithiated by adding 10 mL butyllithium (BuLi, 16 mmol) at a 1:2 ratio (10 mmol PEG + 20 mmol BuLi, MeO-PEG-OLi). Then, 10 times an excess amount (25 g) of α,α'-dibromo-*p*-xylylene (DPX, $C_8H_8Br_2$, MW = 263.96) was added to the lithiated PEG solution and incubated at 65 °C for 24 h with stirring (for a maximum 2–3 days if necessary). At this stage, the solution turned a turbid neon green color and the lithium chloride precipitate (upper side of the vessel) was observed. After the reaction was completed, the solution was precipitated with cold isopropyl alcohol (IPA), centrifuged (8800 rpm, 2 min, −4 °C), the resulting precipitate was solubilized with warm methanol, and then centrifuged (8800 rpm, 2 min, −4 °C) to remove the DPX. The resulting precipitates (DPX impurities) were discarded and the polymer solution was then re-precipitated with IPA and centrifuged (8800 rpm for 2 min at −4 °C). The re-precipitation/centrifugation cycle of the polymer with cold IPA was repeated thrice to purify the polymer, which was then vacuum-dried at room temperature (~25 °C) for 24–48 h. The dried polymer was then dissolved in pure THF (200 mL) under exposure to $N_2$ with stirring in a round-bottomed flask and at this stage, the polymer solution was transparent. The $BrMgSC(=S)C_6H_5$ solution was added immediately to the polymer solution (see supplementary protocol) and incubated at 40 °C for at least 12–72 h with stirring until it was further processed. After the reaction, the mixture was re-precipitated with cold IPA, spun down at 8800 rpm for 2 min at −4 °C, and the re-precipitation/centrifugation cycle was repeated five to eight times until the supernatant became clear. At this step, the resulting precipitate was pink and the polymer was then vacuum-dried for 36 h (yield = 50 g, 100%).

Supplementary protocol: Preparation of $C_6H_5CS_2MgBr$ solution. $PhCS_2MgBr$ was prepared in round-bottomed flask exposed to an $N_2$ atmosphere and in an ice-cold water bath with the following reagents: a) THF, 50 mL; b) carbon disulfide ($CS_2$), 4 mL; and c) phenylmagnesium bromide (PhMgBr) solution, 6.7 mL (3 M in diethyl ether, Sigma-Aldrich, USA). After several minutes, the solution changed to a red color, and the end-point was achieved when the solution turned dark red. After completing the reaction, the ice bath was removed, the solution was stirred for 10–30 min at room temperature (25 °C, the incubation was extended to 1 or 2 h if necessary), and then it was immediately added to the polymer solution.

#### 2.1.2. Synthesis of PEG-b- poly(chloromethylstyrene) (PCMS) block copolymer

Methoxy-poly(ethylene glycol)-*b*-poly(chloromethylstyrene) (MeO-PEG-*b*-PCMS) was synthesized by the radical polymerization of chloromethylstyrene (CMS) using methoxy-poly(ethylene glycol)-hydroxyl (MW = 5000) and the CTA synthesized above (MeO-PEG-$OCH_2PhCH_2SC(=S)Ph$). The polymer backbone of MeO-PEG-*b*-PCMS consisted of PEG and PCMS as the hydrophilic and hydrophobic segments, respectively and the degree of polymerization was 22, as determined using the proton ($^1H$) nuclear magnetic