Reactive Oxidative Species (ROS): Utilizing Injectable Antioxidative Hydrogels and ROS-Producing Therapies to Manage the Double-Edged Sword

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Abstract

Reactive oxygen species (ROS) are generated in cellular metabolism and are essential to cellular signalling networks and physiological functions. However, the functions of ROS are 'double-edged swords' to living systems that have a fragile redox balance between ROS generation and elimination. A modest increase of ROS leads to enhanced cell proliferation, survival and benign immune responses, whereas ROS stress that overwhelms the cellular antioxidant capacity can damage nucleic acids, proteins and lipids, resulting in oncogenic mutations and cell death. ROS are therefore involved in many pathological conditions. On the other hand, ROS present selective toxicity and have been utilised against cancer and pathogens, thus also acting as a double-edged sword in healthcare fields. Injectable antioxidative hydrogels are gel precursors that form hydrogel constructs *in situ* upon delivery *in vivo* to maintain an antioxidative capacity. These hydrogels have been developed to counter ROS-induced pathological conditions, with significant advantages of biocompatibility, excellent moldability, and minimally invasive delivery. The intrinsic, readily controllable ROS-scavenging ability of the functionalised hydrogels overcomes many drawbacks of small molecule antioxidants. The review summarises the roles of ROS in pathological conditions and describes the state-of-the-art of injectable antioxidative hydrogels. A particular emphasis is also given to current ROS-producing therapeutic interventions, providing a potential application of using injectable antioxidant hydrogels to prevent adverse effects of many cancer and infection treatments.

1.Introduction

Oxygen molecules started participating in living metabolisms as early as 2.7 billion years ago, along with reactive oxygen species (ROS) as by-products. ROS are a group of oxygen-containing chemicals that are commonly more reactive than the ground state oxygen[1,2], and have identified as important regulators of many signalling pathways. Moderate levels of ROS are generated during normal cellular metabolic processes and participate in cellular signalling and several cellular functions by reversibly oxidising/modifying protein structure[3,4]. However, uncontrolled ROS have long been known to initiate tumorigenesis, by causing oxidative damage to lipids, proteins and DNA or disrupting oxidative signalling to promote cancer-causing mutations and cell proliferation[4]. Furthermore, several ROS, including superoxide ($O_2\cdot$), hydroxyl radical ($OH\cdot$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$), are involved in many other diseases and conditions (i.e. inflammatory diseases, infection, neurodegeneration, organ failure, cardiac and vascular diseases). These pathological consequences arise from excessive ROS or inadequate intracellular antioxidants disturbing the ROS homeostasis[5], leading to membrane disruption, mitochondrial dysfunction, DNA damage, deregulation of signal pathways, protein up/downregulation, cell cycle arrest and finally apoptosis/necrosis/ autophagy[6].

Some epidemiological evidence exists for this, including the observation that a diet high in natural anti-oxidants is generally associated with better health and a lower incidence of various cancers. Antioxidant food and supplements have therefore been considered weapons to prevent cancer and many diseases for decades[7,8]. However, this firmly held belief has proven to be unsubstantiated by clinical practice using conventional antioxidants under complicated physiological and pathological conditions[9]. Local administration of small molecule antioxidants, such as sodium sulfite, potassium sulfite, sodium bisulfite, and sodium metabisulfite, were utilised to alleviate overproduced ROS that are harmful to the human body[10–12]. However, the inhibition of bioactivity and uncontrollable stability of the conventional antioxidants decreases the efficacy of those approaches, although some of them have achieved approval regarding safety[10–12].

To overcome the abovementioned drawbacks of small molecule antioxidants, injectable hydrogels containing functional groups acting as ROS scavengers were developed. Hydrogels consist of physically or chemically crosslinked hydrophilic polymers and a significant amount of water retained within the 3D polymeric networks[13]. Due to their superior biocompatibility, diverse and flexible fabrication with infinite combinations of monomers and favourable physical characteristics, hydrogels have been extensively used in various biomedical applications, alone, or loaded with therapeutic cargos (i.e. small molecules, macromolecules and cells)[13,14]. As one of the major goals of hydrogel-based technology, injectable hydrogel, polymeric gel precursors that can be injected and in situ crosslinked to form hydrogels *in vivo*, have attracted increasing attention and developed many drug delivery and tissue engineering applications[14–20]. Relying on the diversification of in situ formation mechanisms, vast combinations of functional polymers and minimally invasive implantation, injectable hydrogels are capable of meeting specific requirements for various pathological conditions. A class of injectable antioxidative hydrogels has thus been developed with promising therapeutic efficacy in ROS homeostasis-related diseases[1,10,21–23].

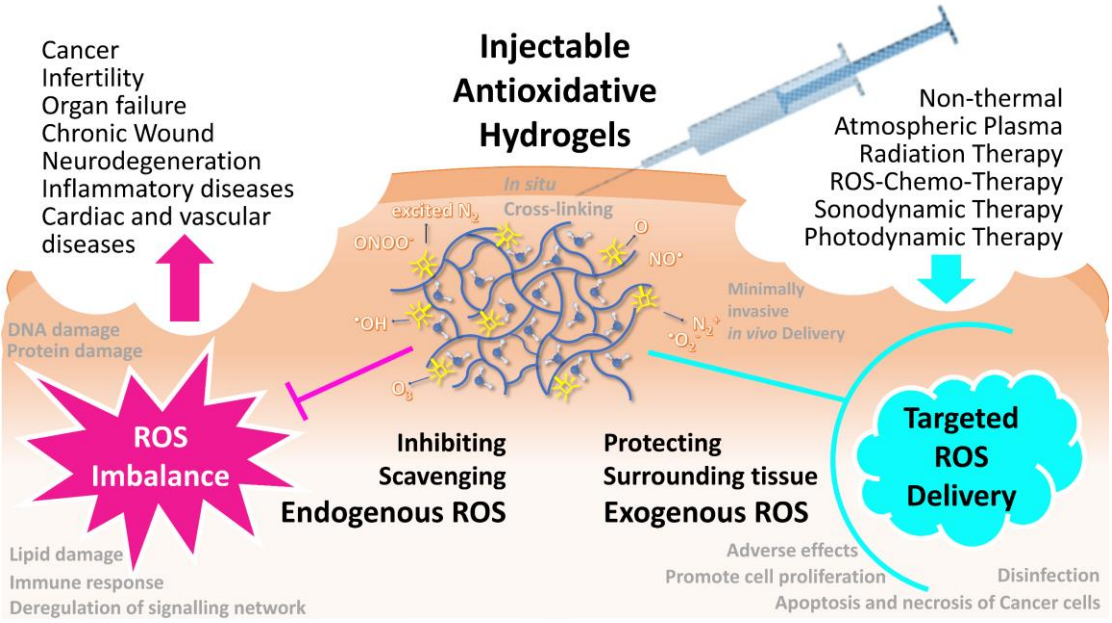


Figure 1. *Schematic illustration of the structure of this review.*

The organization and scope of the review are exhibited in Figure 1. In this review article, the roles of ROS in pathological conditions were summarised, and the recent developments of injectable antioxidative hydrogels were comprehensively introduced. Meanwhile, special attention was given to anti-cancer or anti-infection ROS-producing therapeutic interventions, whereas the leakage of ROS can induce adverse effects but may be preventable by the application of injectable antioxidative hydrogels.

2. Endogenous ROS generation in pathological conditions

ROS are generated in almost all types of metabolically active cells. Although the majority of ROS production takes place in the respiratory chain by oxidative phosphorylation in normal mammalian cells, and in glycolysis and lactic acid fermentation, which is generally increased due to the enhanced metabolism in cancerous cells[24,25], the detailed mechanisms of ROS generation are still not fully understood. However, the importance of ROS homeostasis to normal physiologic functioning has been thoroughly proven, and the causes of imbalance between ROS production and ROS elimination in many pathological conditions have been revealed (Figure 2).

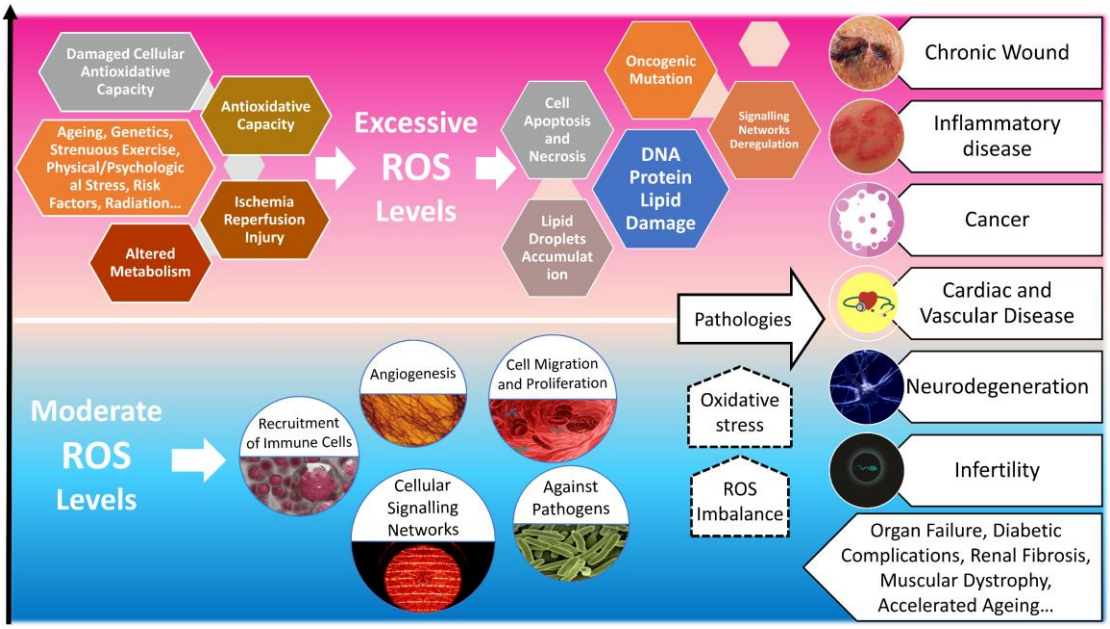


Figure 2. *Schematic illustration of the summarised roles of ROS in physiologic and pathological conditions.*

2.1 ROS generation in wound healing

The healing processes in response to normal tissue injury can be divided into 4 parts, including hemostasis, inflammation, proliferation and maturation, which are not

strictly and explicitly in distinguishable chronological order, but more overlapping[1]. 1) Hemostasis is the first process after injury and bleeding. Mechanical support for the injured tissue is provided by the coagulation of exudates. 2) Then debris are removed by inflammatory cells, monocytes, lymphocytes, and macrophages, in the second stage, to prepare wound beds for the construction of granulation tissue. 3) At the third stage, the proliferation of epithelial cells and fibroblasts takes place in the injured area and gradually fills the surface of the open wound with granulation tissue. 4) The formation of connective tissue and enhancement of the new epithelium is involved in the final stage, maturity, also known as remodelling[1]. During those stages, a moderate level of ROS is known to stimulate cell migration and angiogenesis, and thus promote normal wound healing[1,26]. ROS act as secondary messengers in coordination with: (i) the recruitment of lymphoid cells to the injured site, (ii) the promotion of effective repair via non-lymphoid cells and (iii) the optimal perfusion of blood into the healing area via regulated formation of blood vessels. ROS also function in the immune defence through phagocytes that generate a ROS burst targeting pathogens existing in wounds for bacteriostatic effects[27].

However, despite the pivotal role of ROS in the wound healing response, oxidative stress can be induced in would healing processes when the increasing of levels of ROS overwhelm the activity of intercellular and intracellular antioxidant enzymes and scavengers, leading to stalled wound healing, and subsequently chronic wounds. Clinically, many chronic wounds are caused by sustained stimulation, such as infection, repeated tissue damage, hyperglycaemia or persistent inflammatory reactions, which interfere with physiological healing mechanisms[1]. For instance, it is noted that the infection and persistent hyperglycaemia in diabetic chronic wounds enhance the levels of advanced glycation end products in the blood, leading to excessive ROS accumulation, while the inflammation response activated by chronic wounds also generates a large amount of ROS[1,28]. Thus, the toxic level of ROS then causes deregulation of cellular functions, changes of gene expression, apoptotic or necrotic cell death and further damage to the injured tissue. Interestingly, the use of H_2O_2-infused cream attributed to angiogenesis and increased blood flow to the ischaemic ulcers in Guinea pigs[27]. Moreover, a comparison between 10 mM and 166 mM H_2O_2 in phosphate-buffered saline (PBS) for excisional wounds treatment was performed in mice. 166 mM H_2O_2 delayed would closure, whereas 10 mM showed minor effects on wound closure but improved angiogenesis[29]. Considering the

'double-edge sword' property of ROS, the direct and complete removal of ROS by local administration of conventional antioxidants may lead to failure of treatment and a controllable ROS scavenger capacity that can be achieved by injectable antioxidative hydrogels is desirable. For example, small molecule antioxidant activity can only be manipulated by modifying the dose. In contrast, poly(β-hydrazide ester) hydrogels possessing anti-ROS functional disulfide components[10], can be tailored by changing concentration, molecular weight and monomer ration, giving more flexibility in treatment. More importantly, due to the dynamic ROS level of the targeted lesion, an over-dosing of conventional small molecular antioxidants may lead to a lack of ROS for angiogenesis and other bioactivities. However, the degradation of the hydrogel manipulated by the ROS level may lead to a smart response to maintain a moderate ROS level for optimal treatment[10].

2.2 ROS generation in chronic inflammatory diseases and cancer

In addition to the abovementioned roles of ROS in the immune system and their effects on cells, disordered ROS levels are also considered to be responsible for the association between chronic inflammatory diseases and increased tumour incidence[30]. At the initial stage, ROS overproduction, induced by ageing, genetics, strenuous exercise, physical and/or psychological stress, traditional risk factors (i.e. smoking and air pollution) and harmful rays or radiation (i.e. UV and ionising radiation), along with reduced ROS scavenging ability in the human body, can cause the development of inflammatory disorders[31,32]. Initiation of inflammatory responses leads to the recruitment and functioning of infiltrating myeloid cells, including macrophages, neutrophils, or their precursors that response to ROS or/and further generate ROS. The ROS homeostasis is then disrupted, and the ROS level accumulates like a 'snowball', until cell death or an unstable balance between antioxidant level and ROS. With the loss of homeostasis, the ROS which were supposed to provide protective effects by inducing programmed cell apoptosis or by enhancing T cell responses, are responsible for increased cancer incidence. This occurs through the mutagenesis of healthy cells, as ROS have been shown to induce damage of DNA, lipid and proteins[6,30,33,34]. Many cancer types can be potentially caused by inflammation and ROS-related mutagenesis. For example, Canli *et al.* demonstrated that the amplified ROS generation by myeloid cells can promote intestinal mutagenesis[35]. The infection of bacterium *H. pylori* in the human stomach was found to trigger a chronic

inflammatory response, which is the major risk factor for gastric cancer development[36]. As the largest organ protecting the human body, skin can be often exposed to external ROS sources (i.e. ozone and other pollution) and many inducements causing ROS generation (i.e. UV and radiation). Also, heterogeneous chronic wounds are considered to be major aetiological factors in skin cancers.

On the other hand, skin is one of the easiest targets for ROS or anti-ROS treatment, such as H_2O_2 wound treatment or common antioxidants (i.e. Vitamin C and Vitamin E) used in skin care products, The nature of topical treatment allows more efficient *in vivo* study for exploring the roles of ROS in skin cancers. The various origins and types of skin cancer also could give more comprehensive understanding of ROS in mutagenesis and metastasis. For example, it was demonstrated that malignant melanoma cells may increase oxidative stress to damage surrounding tissue to support metastasis. In non-melanoma skin cancer, diminished antioxidant defence caused by chronic UV exposure was suggested to contribute to multistep carcinogenesis[37].

Interestingly, cancer cells are generally sensitive to low doses of exogenous ROS treatment and can subsequently undergo apoptosis compared to normal cells. These doses often do not produce any measurable toxic effect on corresponding normal cells[38–40]. The wide range and high amounts of ROS generated were proposed to play a major role in the selective cytotoxicity[41]. Exogenous ROS can further stimulate cancer cells to produce more endogenous ROS[38]. Evidence shows that a higher level of ROS was generated in cancer cells, compared to normal cells, due to higher metabolic activity and more rapid proliferation of transformed cells[42]. Hence, the cellular antioxidant system works under more pressure in tumour cells to protect them from oxidative stress, suggesting it may be possible to selectively eliminate them with inducers of tumour ROS[43]. One hypothesis for the increased production of ROS in tumour cells is the difference in metabolism between normal and cancer cells. Cancer cells carry out more biomass synthesis per unit time due to the unregulated and relatively rapid cell growth and proliferation[44]. It has been found that through a phenomenon called “the Warburg effect”, cancer cells rely primarily on glycolysis and lactic acid fermentation to generate energy, whereas other nucleated cells generate most adenosine triphosphate (ATP) by oxidative phosphorylation[24]. To deal with higher intracellular ROS levels, tumour cells synthesise nicotinamide adenine dinucleotide phosphate (NADPH). NADPH is utilised as a reducing equivalent to reduce

thioredoxins (TRX), peptides that in turn reduce oxidised proteins such as peroxiredoxins, a family of hydrogen peroxide-scavenging enzymes[45]. NADPH is also involved in the generation of the antioxidant glutathione (GSH), an important tripeptide in antioxidant systems[46]. Despite the increased expression of antioxidant systems, cancer cells generally have a higher baseline intracellular ROS, which makes it more difficult for the antioxidant capacity of tumour cells to deal with additional oxidative stressors[44]. In this case, ROS act also as a double-edged sword to cancer cells and have been utilised in many interventions for tumour treatment. With the artificially overproduced intracellular levels of ROS, lipids, proteins, and DNA can be damaged in cancer cells, leading to lipid peroxidation-initiated oxidative stress, inhibition of phosphatases, alteration of cytoplasmic and nuclear signalling, disruption of epigenetic modulators, etc., and eventually apoptosis, autophagy or ferroptosis of cancer cells[4]. However, the leakage of ROS into surrounding healthy tissue and the circulatory system is one of the major causes of side effects from cancer treatment, which indicates a new potential use of injectable antioxidative hydrogels.

2.3 ROS generation in cardiac and vascular diseases

ROS signalling has proven to play an important role in the functional crosstalk among cardiomyocytes, endothelial cells and fibroblasts in heart tissue, which influences contractile function, cardiomyocyte growth, hypertrophy, angiogenesis, and fibrosis[47]. Since ROS control normal heart function, disrupted ROS levels are related to many cardiac pathologies. In the decompensated heart, the activity of antioxidant enzymes is decreased, suppressing the scavenger defence against oxidative stress, leading to an increase of ROS levels. ROS have been reported to be involved in cardiac hypertrophy, atherosclerosis, myocardial ischemia/reperfusion injury, and heart failure[48]. For instance, the most common cause of heart failure is myocardial ischaemia, which leads to ischaemia–reperfusion injury and subsequent accumulation of ROS and apoptosis of cardiomyocytes[49]. Similarly, insufficient heart function triggers cardiac hypertrophy for compensation but also increases ROS levels in response to enhanced energy demand[50]. The ubiA prenyltransferase domain-containing protein 1 (UBIAD1) can reduce oxidative damage via antioxidant Coenzyme Q10 to protect cardiovascular function[51]. Injectable antioxidative hydrogels have been tested *in vitro* for suppression of oxidative stress damage in cardiomyocytes[22], which are introduced in detail in the next section. Like their pivotal role in cardiac functions,

ROS regulate angiogenesis via their interference with vascular cell proliferation and apoptosis and are essential for some vascular activity, such as relaxation of cerebral arteries[52]. Despite the beneficial effects of ROS from their functions as signalling messengers and in responding to energy demand, overproduction of ROS still leads to many vascular pathologies. Endogenous or exogenous oxidative stress could trigger hypertension and stimulate its pathological process[5]. Ischaemia–reperfusion cause not only oxidative damage to cardiomyocytes but also to affected blood vessels and surrounding tissues. Retinal dysfunction is caused when the blood pressure is lowered by the reperfusion injury and associated ROS overproduction, and similar damage also occurs in the brain, kidney, testis and other organs and tissues[53]. Despite the fact that no specific treatment for the prevention or recovery of the high morbidity and mortality associated with ischaemia–reperfusion injury is available, regulating ROS level has proven to reduce the injury in pre-conditioning protocols[53]. It was also reported that overproduction of ROS regulated atherosclerosis via triggering lipid peroxidation and interfering with macrophages[54], promoting thrombus formation in arteries[55,56], and inducing pulmonary vascular lesions and inflammation[5,57].

2.4 ROS generation in neurodegeneration and other diseases

Due to the long-lifespan or post-mitotic nature of neuron cells, they are vulnerable to oxidative stress caused by overgeneration of ROS or impairment of antioxidative capacity, leading to mitochondrial dysfunction and initiation of the cell death cascade[58]. It has been shown that many neurodegenerative diseases and a number of neurological conditions, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, neurodegeneration with brain iron accumulation and Huntington's disease, are associated with ROS overproduction and mitochondrial redox imbalance[58]. Mitochondrial defects have proven to induce ROS overproduction and thus trigger c-Jun-N-Terminal Kinase (JNK) and Sterol Regulatory Element Binding Protein (SREBP) activity in neurons, which further cause the accumulation of lipid droplets and subsequently neurodegeneration[59]. Antioxidants were found to be able to rescue the ROS-induced apoptosis of neurons from Down syndrome patients *in vitro* and delay neurodegeneration in animal models with ROS/lipid droplets[59,60]. ROS and downstream signalling pathway inhibitors, such as the PPARγ coactivator 1α (PGC-1 α) transcriptional coactivators[61] and ferritin heavy chain (FHC)[62], have been demonstrated to reduce ROS levels and protect neurons from apoptosis. However, it

should also be noted that the presence of ROS in neurons is vital for their development, and is essential for synaptic plasticity and memory formation, and they play a fundamental role in metabolism and energy perfusion[16].

In addition to the abovementioned pathological conditions, ROS have proven to be responsible for many other diseases or physiological disorders, including organ failure[5], diabetic complications[63], renal fibrosis[64], muscular dystrophy[65], accelerated ageing[66] and infertility[67].

2.5 Current treatment strategies and limitations for excessive ROS

The mechanisms of antioxidant defence include prevention, interception and repair. Prevention is the first line of defence against oxidative stress, which can be conducted by maintaining a healthy lifestyle and avoiding ROS-triggering factors, such as pollution and UV radiation[31,32], although the benefit of natural antioxidant uptake from food and supplements is still controversial[9]. Thus, the interception of ROS overproduction in pathological conditions and the subsequent repair are key points in treatment of ROS-related diseases.

Many natural or synthetic chemicals have proven to have antioxidative properties and are safe to use *in vivo*, such as ascorbate (vitamin C), β-carotene, flavonoids, glutathione, N-acetylcysteine, tocopherol (vitamin E), taurine, and hypotaurine[9]. Therefore, the current major treatment strategy for the interception of excessive ROS is systemic or local administration of those small molecule antioxidants. However, although these antioxidant treatments can show promising benefits in *in vivo* results from animal models of diseases or aging[68–71], using antioxidants against ROS-related diseases presents no or limited improvement in many clinical trials. For instance, the treatment of adult respiratory distress syndrome (ARDS) with N-acetylcysteine (150 mg/kg as a loading dose and then 20 mg/kg/hr for 6 days) demonstrated no benefit compared to the control group, while the problems with N-acetylcysteine and coagulation need to be further elucidated[72]. The treatment of Alzheimer's disease using antioxidants, including β-carotene, vitamin C, vitamin E and flavonoids (manual interventions, such as using vitamin E 200–800 IU/d or 2000 IU/d, or determined by food frequency questionnaires), have been conducted in several clinical studies, but no conclusive positive answer was obtained, possibly due in part to inappropriate timing of administration, blood-brain barrier (BBB) permeability, or insufficient drug levels at the targeted area in central nervous system[73]. The use of antioxidant

supplements including glutathione and vitamins E and C in clinical trials for the treatment of male infertility are also controversial, despite the clear role of excessive ROS in infertility[74]. One report showed that the seminal ROS Log (ROS+1) increased from the control group (1.39±0.73) to infertility patients (i.e. 2.65±1.01), while the total antioxidant capacity (TAC, Trolox equivalent) in seminal plasma decreases from controls (1650.93±532.22) to infertility groups (i.e. 1051.98±380.88)[74]. Factors influencing those results include poor stability of the antioxidants, suboptimal doses and anti-ROS capacity, nonspecific delivery and off-target effects. Thus, new and more powerful antioxidants, the efficiency of delivery, elimination of off-target effects and controllability of the antioxidative ability are essential for the successful practice of antioxidative treatment. Therefore, injectable antioxidative hydrogels, as they have unique advantages compared to conventional antioxidants, non-injectable antioxidative hydrogels or other anti-ROS strategies, have been developed or have great potential for the treatments of a variety of ROS-related diseases and pathological conditions, which are introduced in the next section.

3. Recent development of injectable antioxidative hydrogels

Excellent *in vivo* and clinical outcomes were presented using antioxidative hydrogels for chronic wound healing, as the advantages of loading antioxidants with hydrogels or using self-antioxidative hydrogels include steady ROS-scavenging abilities, high biocompatibility, and tuneable physicochemical properties for wound healing[1]. However, there are several drawbacks of conventional hydrogels in tissue engineering which have driven the investigation of new technologies for hydrogel fabrication, such as the need for toxic crosslinkers, complicated production methods, poor biocompatibility, uncontrolled shape, and requirement of complicated surgical implantation.[75,76]

In particular, injectable hydrogels formed by *in situ* crosslinking, which facilitates the transition from an aqueous mixture of gel precursor to a solid gel, have attracted much attention.[76] Injectable hydrogels possess almost all the advantages of hydrogels in biological and biomedical applications and their own unique properties, which makes them more promising.[77] The major advantages of injectable hydrogels over the conventional prefabricated hydrogels include reduced cell damage during injection, easy manipulation during the applications, and minimally invasive procedures.[14,78] Moreover, injectable hydrogels are very effective and of great value when transplanting and localising cells to a desired anatomic site. Thus, injectable hydrogels have emerged as the most promising biomaterials in recent years[10,18,79–82]. The key technology to achieve in situ formation of hydrogels is the physical or chemical crosslinking taking place in the human body, which can precisely mimic the properties of the native tissues[15,83]. Due to the diversity of hydrogel materials, the crosslinking abilities and the properties of injectable hydrogels vary by design. Usually, analysing storage moduli (G') determined by rheometry is the standard method used to evaluate the gelation and physical properties of the hydrogel during and upon crosslinking. For example, 150 Pa of G' measured at the frequency of 1.0 Hz and a fixed strain of 1% represented a soft hydrogel, whereas G' around 80 Pa represent viscous liquid due to insufficient crosslinking degree[79]. Many well-studied hydrogels, such as hydrogels of cellulose derivatives including carboxymethylcellulose, chitosan or poly (ethylene glycol), can be used as benchmarks for *in-situ* crosslinking studies. Many *in-situ* crosslinking methods were investigated, briefly, the crosslinking mechanisms of the injectable hydrogels include physical-related reactions in response to environmental stimuli (i.e. temperature, pH and ionic strength) and chemical reactions including

Michael-type addition reaction, disulfide bond formation, photopolymerisation, Schiff-based gelation, and enzyme-triggered reactions[83,84]. Qian et al. reported an injectable and self-healing hydrogel crosslinked by dynamic covalent bonds and subtly designed to possess five responsive properties (Figure 3, 4)[79]. The hydrogel showed an on-demand degradation profile and a significantly higher lap shear strength than BioGlue®, a well-known commercial adhesive product. Therefore, this novel injectable hydrogel can function as a potent multi-responsive tissue adhesive[79].

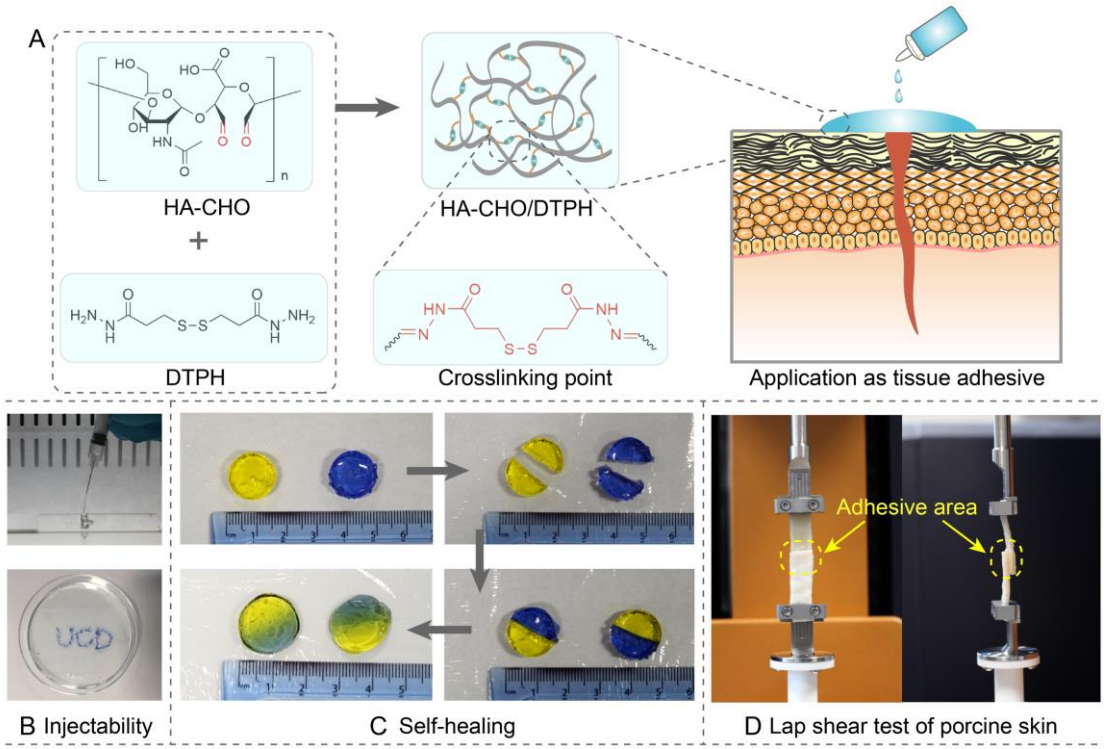
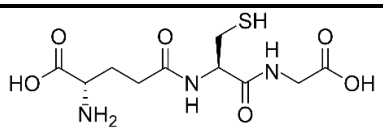
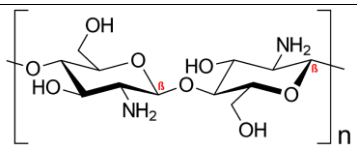
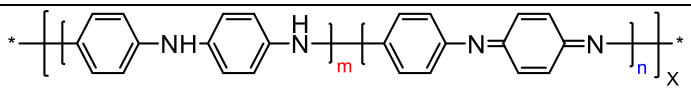
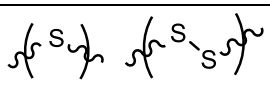
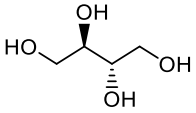


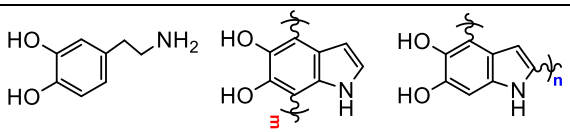
Figure 3. *(A) Schematic illustration of fabrication and application of the aldehyde functionalised hyaluronic acid /disulfide containing crosslinker-3,3'-dithiobis(propionic hydrazide) (HA-CHO/DTPH) hydrogel as a tissue adhesive. (B) Injectability of HA-CHO/DTPH hydrogel. The hydrogel was able to pass through a 28-gauge needle. The hydrogels were stained with trypan blue for observation. (C) Self-healing process of the HA-CHO/DTPH hydrogel. Complete healing occurred in 2 h. The hydrogels were stained yellow and blue for observation. (D) Representative images of tissue adhesive strength determined by the lap shear test of porcine skin. Reproduced from ref. 76 with permission.*

Increasing attention has been given to utilising injectable hydrogels to overcome the drawbacks of using traditional antioxidants for ROS-related pathological disorders, by including ROS-sensitive functional groups, such as dopamine, sulfide, disulfide, poly(thioketal), and poly(propylene sulfide), in the formation of the hydrogel precursors[85–87]. Many hydrogels loaded with natural (i.e. natural polyphenols or red jujube extract) or modified antioxidants (i.e. modified polyphenols) have been reported[1,88–90]. Furthermore, several novel injectable hydrogels loaded with

antioxidants or antioxidative nanocomplexes, such as baicalin/F127 hydrogel[91], mitochondria-targeted antioxidant Mito-2,2,6,6-tetramethylpiperidine-N-oxyl loaded in self-assembling peptide[92], Ferulic acid loaded hydrogel[93], curcumin loaded quaternized chitosan/benzaldehyde-terminated Pluronic®F127 hydrogel[94] and fullerenol antioxidative nanoparticles (NP) /alginate hydrogel[95], have shown potential therapeutic antioxidative efficacy. However, the possible release instability of antioxidant components *in vivo*, overly abundant but unpractical choices of antioxidants, and infeasibility or complexity of fabrication and combination have been considered. Therefore, the focus of this review article is on injectable hydrogels with sustained self-antioxidative capacity. The stability of small molecule antioxidants is difficult to control and may lead to over-suppression of ROS levels in the targeted area, surrounding tissue and/or blood vessels, causing inhibition of bioactivity as side effects[12], whereas injectable antioxidative hydrogels combine the advantages of in situ gelation and spatiotemporally tuneable antioxidative capacity, and thus can be favoured for the treatments of ROS-related diseases. The development and status of injectable antioxidative hydrogels in biomaterial and biomedical applications is remarkable according to the latest research results[96]. A few representative anti-ROS functional groups for hydrogel fabrication were briefly summarised in Table 1.

Table 1. Representative ROS-scavenging functional groups/moieties.

Name	Structure	Ref.
Glutathione		22
Chitosan		97,98
Aniline tetramer, Polyaniline		23,99–101
Sulfide, Disulfide		10,102
Sugar alcohols		103

Dopamine, Poly Dopamine		[21, 85, 104]

Lee *et al.* reported hydrogels composed of chitosan and eugenol with good antioxidant activity many years ago, which laid a foundation for utilising antioxidant hydrogels in chronic wounds[1]. Many ROS responsive polymers for not only wound repair but various biomedical applications have been developed, reported and summarised, such as poly(propylene sulfide), selenium-containing polymers, tellurium-containing polymers, poly(thioketal), phenylboronic acid/ester-containing polymers, poly(L-

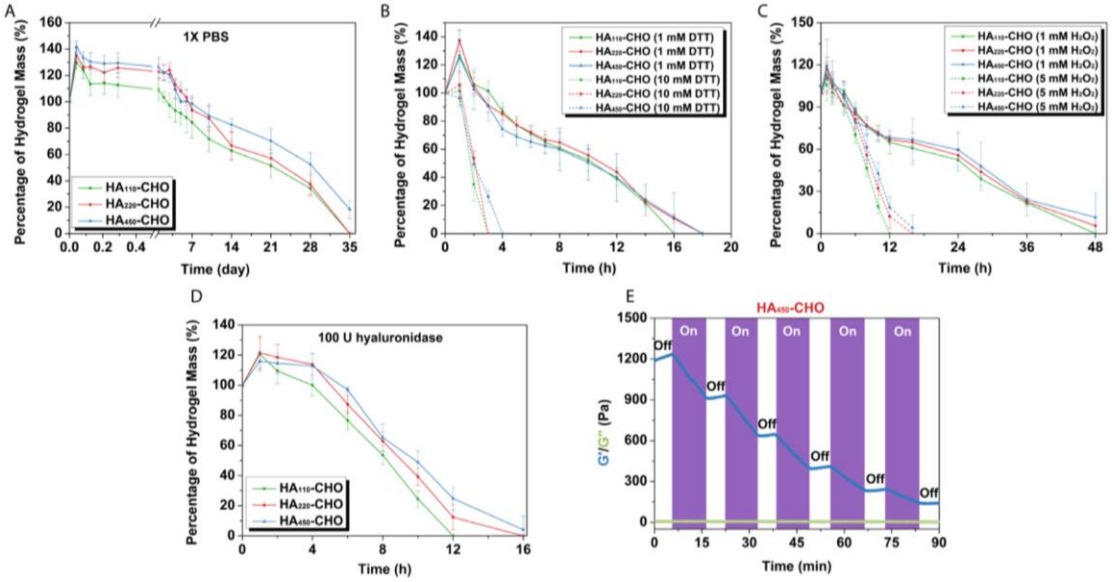


Figure 4. *(A) Control groups of chemical-responsive degradation profiles with 1X PBS buffer. (B) Reduction-responsive degradation profiles with a concentration of 1 mM and 10 mM of dithiothreitol (DTT). (C) Oxidation-responsive degradation profiles with a concentration of 1 mM and 5 mM of H2O2. (D) Hyaluronidase degradation profiles. (E) UV-responsive degradation presented by an intermittent decrease of storage modulus of HA450–CHO/DTPH hydrogel. Reproduced from ref. 76 with permission.*

methionine) and poly(L-proline)[87]. Although the development of injectable antioxidative hydrogels is facing some key challenges, such as hazardous reagents, complicated synthetic procedures, limited and low-compatibility mechanical properties, and inefficient antioxidative efficacy[105], many promising injectable antioxidative hydrogels are recently reported.

Many functional groups or moieties were reported to possess good ROS scavenger capability when they are utilised in injectable hydrogels as side chains, backbone or copolymerisation blocks. Li *et al.* developed a thermosensitive chitosan chloride-glutathione (CSCl-GSH) hydrogel to suppress oxidative stress damage in cardiomyocytes[22]. The antioxidant glutathione (GSH) was conjugated on the chitosan

chloride chain. It has been shown that the CSCI-GSH conjugates successfully retained the effective ROS scavenger properties from GSH, along with excellent biocompatibility to support the growth of cardiomyocytes *in vitro*, which offer the promising potential for myocardia repair[22]. Chitosan also has self-antioxidant properties, presenting anti-inflammatory effects and thus contributing to heart and blood vessel repair[97]. A polypyrrole (PPy)-chitosan was also reported to be capable of maintaining myocardial function via increased electrical conductivity and antioxidant capacity[98].

The tetraaniline (TA), also known as aniline tetramer (AT) block in carboxyl tetraaniline-poly(D, L-lactic acid-*co*-glycolic acid)-poly(ethylene glycol)-poly(D, L-lactic acid-*co*-glycolic acid)-carboxyl tetraaniline (CTA-PLGA-PEG-PLGA-CTA) copolymers were found to remain the redox-active nature while the antioxidant property of the α-cyclodextrin co-constructed supramolecular hydrogels increases with the increasing proportion of TA block[99]. Besides the antioxidant property, good biocompatibility of the materials *in vivo* and the proliferation acceleration of encapsulated cells via electrical stimuli were also reported, but relatively fast gel erosion and poor mechanical property were also noted[99]. Later on, fabrication of antioxidant hydrogels by mixing the biocompatible polymer N-carboxyethyl chitosan (CEC) and oxidised hyaluronic acid-graft-aniline tetramer (OHA-AT) polymer was also reported, presenting good antibacterial properties with the addition of amoxicillin. More importantly, this combination accelerated wound healing in a full-thickness skin defect model, including higher granulation tissue thickness, collagen disposition and more angiogenesis, which is associated with the antioxidative properties from the addition of aniline tetramer[100]. A series of injectable antioxidative hydrogels based on quaternized chitosan-g-polyaniline (QCSP) and benzaldehyde group functionalised poly(ethylene glycol)-co-poly(glycerol sebacate) (PEGS-FA) were developed. Polyaniline components contributed to the robust antioxidative properties, along with an optimal crosslinker concentration of 1.5 wt% PEGS-FA presenting excellent blood clotting capacity, leading to significantly enhanced *in vivo* wound healing [23]. Similarly, a gelatin-graft-polyaniline/periodate-oxidised alginate hydrogel was reported for potential application of injectable electroconductive and antioxidative hydrogels in neural tissue engineering[101]. Whereas polyaniline provided the antioxidative abilities, the branched polyethyleneimine (PEI) was introduced to tune the properties of

hydrogels, and Schiff's base linkages and ionic interactions were applied for the crosslinking of hydrogels[101].

Nitroxide radicals were utilised as ROS scavenging side chains in the polyamine-PEG-polyamine triblock copolymer, which was then complexed with poly(acrylic acid) to prepare redox flower micelles exhibiting gelation under physiological conditions[106]. *In vivo*, this injectable redox gel has proven to be efficient for preventing the formation of postsurgical tissue adhesions by dramatic inhibition of inflammation and oxidative stress, especially suppression of the increase of white blood cells level, thus preventing local inflammation from spreading to the entire body[106].

Sulfide reacts irreversibly with ROS, thus it can be utilised to impart inherent antioxidant capacities to the injectable hydrogel system. A dual thermo- and ROS-responsive hydrogel comprising the ABC triblock polymer poly[(propylene sulfide)-block-(N,N-dimethyl acrylamide)-block-(N-isopropylacrylamide)] was reported[102]. The poly(propylene sulfide) block presented anti-ROS properties, resulting in significantly enhanced cytoprotection of mesenchymal stem cells (MSCs) and insulin-producing β-cell pseudo-islets against ROS toxicity when the cells were encapsulated in the hydrogels integrated with type 1 collagen, which is promising for the improvement of cell therapies[102].

The use of disulfide in the injectable hydrogels was also demonstrated. Xu *et al.* reported a simple and direct synthetic procedure, using non-hazardous reagents, for the fabrication of a novel class of hyperbranched poly(β-hydrazide esters). Poly(ethylene glycol)diacrylates (PEGDA) with various molecular weights were selected to form macromers via Michael addition approach with 3,3'-dithiobis(butanoic hydrazide) (DTP) or suberic dihydrazide (SDH) as non-disulfide control monomer[10] (Figure 5). The hydrogels with thiolated hyaluronic acid crosslinked through thiol–ene chemistry at physiological conditions or by UV radiation with photoinitiators, presenting excellent radical scavenging ability, biocompatibility and mechanical properties, which can be used for a variety of biomedical applications[10]. Other sulfide-containing polymers were also applied in the preparation of injectable antioxidant hydrogels. 2,2'-(Ethylenedioxy) diethanethiol (EDDT) and 2,2-dimethoxy propane (DMP) were utilised to synthesise EDDT-poly(thioketal) (PTK) dithiol polymers via thiol-maleimide chemistry, which were then crosslinked with maleimide-functionalised PEG (PEG-MAL) macromers to form antioxidant

hydrogels[107]. The PTK hydrogels delivered strong anti-ROS protection for an enhanced encapsulated mesenchymal stem cell retention and viability[107]. Similarly, a class of

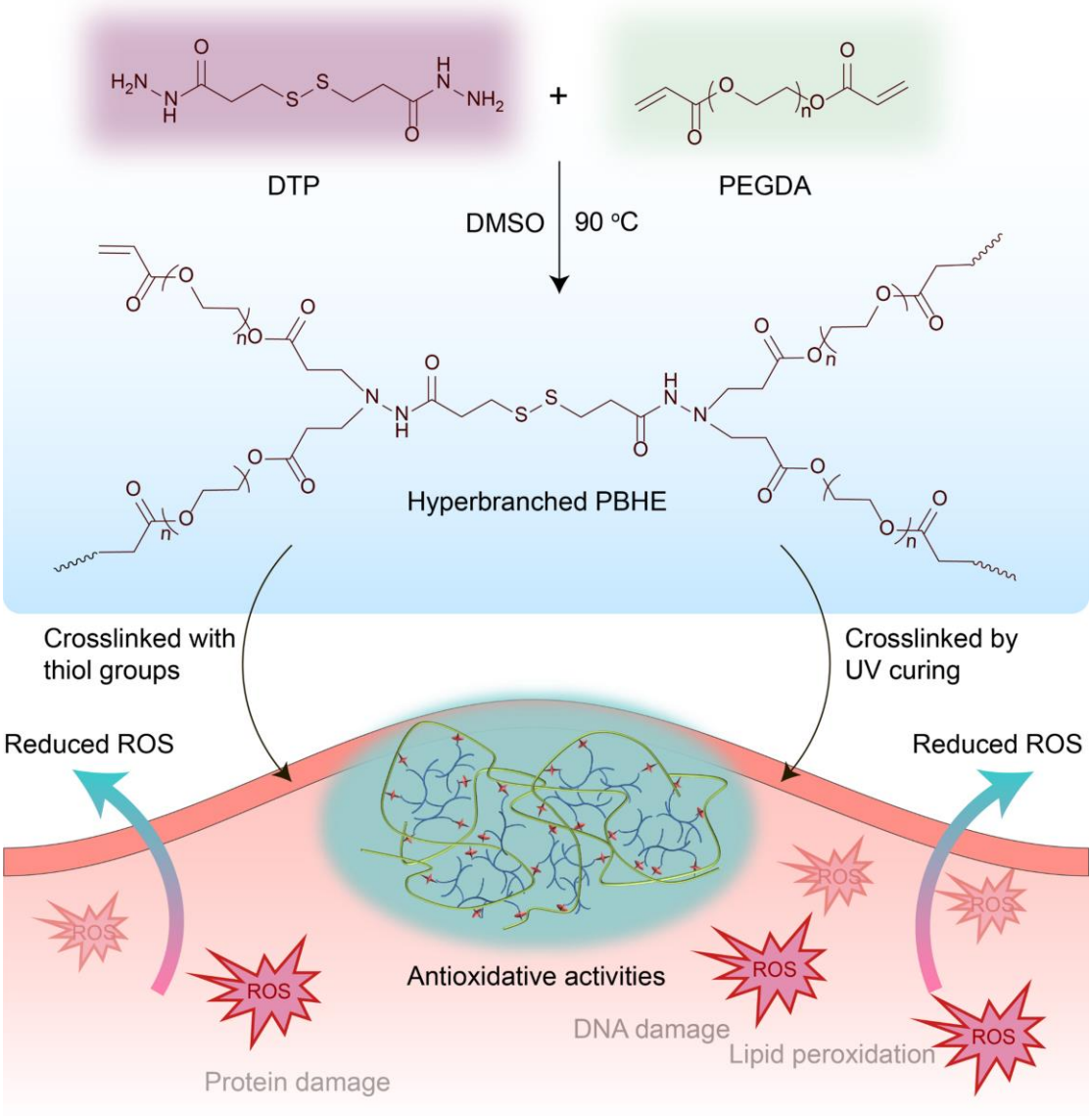


***Figure 5**. Example scheme for the design of an antioxidative injectable hydrogel. Reproduced with permission from ref. 10. Copyright (2018) American Chemical Society.*

polymers containing thioketal linkages has been reported for excellent myocardial infarction therapeutic effect in vivo[108]. Thioketal diethyl amine and polyethylene glycol diacrylate were used to synthesise the ROS-scavenging polymers, which were then copolymerized with methacrylate hyaluronic acid to form a UV-response hydrogel[108]. In addition to the ROS-scavenging ability, a commercially available and biocompatible catalase (CAT) that can turn H_2O_2 to O_2, was also entrapped during the hydrogel formation and presented excellent treatment effects combined with the ROS-scavenging hydrogels[108].

Sugar alcohols present free radical scavenging abilities. Thus the free hydroxyl group in sugar alcohols can be integrated into polymers for antioxidative hydrogels[103]. Komeri *et al.* reported a hydrogel prepared with D-xylitol-*co*-fumarate-co-poly ethylene adipate-*co*-PEG comaromer and PEGDiacrylate which can scavenge ROS, a property possibly relying on the free π electrons associated with uncrosslinked fumarate bonds, or hydrogen atoms associated with sugar alcohols/PEG[103].

Dopamine, as a natural bioactive molecule, possesses good antioxidant abilities. A photocrosslinkable dopamine-containing poly(β-amino ester) (DPAE) synthesised by Michael type addition between dopamine hydrochloride and poly(ethylene glycol) diacrylate (PEGDA) was reported, resulting in robust radical scavenging efficacy but was not toxic to dopaminergic SH-SY5Y cells as well as primary astrocytes and primary embryonic rat ventral midbrain cultures[85]. Polydopamine, along with modified dopamine, are also applied for the synthesis of injectable antioxidative hydrogels. Gelatin-grafted-dopamine (GT-DA) and polydopamine-coated carbon nanotubes (CNT-PDA) were reported to engineer injectable GT-DA/chitosan/CNT composite hydrogels with multiple properties, including antibacterial, adhesive, antioxidant and conductive abilities, via an H_2O_2/horseradish peroxidase (HRP) catalytic system oxidatively coupling catechol groups[21]. The potent antioxidative properties from the catechol group and polydopamine, along with other bioactive functions, were found to endow the materials with potential as excellent wound healing dressings[21]. Similarly, reduced graphene oxide (rGO), hyaluronic acid-graft-dopamine and a H_2O_2/HRP system were used to prepare a series of adhesive hemostatic antioxidant conductive hydrogels for wound dressing[104]. Upregulation of CD31 (growth factor) and improvement of the granulation tissue thickness and collagen deposition contributed to vascularisation, which was associated with the antioxidative properties of these hydrogels[104].

Among the diversified monomers and structures for the inherent anti-ROS ability of injectable hydrogels, varying degrees of ROS scavenging has been demonstrated to be achieved by adjusting the design of polymerisation. However, due to the diversity of the materials, standard *in vitro* experiments for determining the antioxidative capacity are crucial to efficiently evaluate and screen the functions of developed hydrogels before further *in vivo* and preclinical tests. Swelling and degradation profiles of hydrogels incubated in PBS and H_2O_2 solutions (0.1-1 mM) can be used to demonstrate their potential antioxidative biomedical application (Figure 6A-D), as the interaction

between ROS and hydrogels may manipulate the degradation behaviour. 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging assay can be used to determine the antioxidative property of hydrogels quantitatively. The DPPH radical scavenging efficiency calculated by measuring absorbance at 517 nm can be used to compare the anti-ROS abilities of hydrogel candidates, while EC_{50} is determined for precise comparison (Figure 6E-H). When the antioxidative ability and biocompatibility of designed hydrogels have been proved to be at desired levels, then *in* vivo analysis can be undertaken.

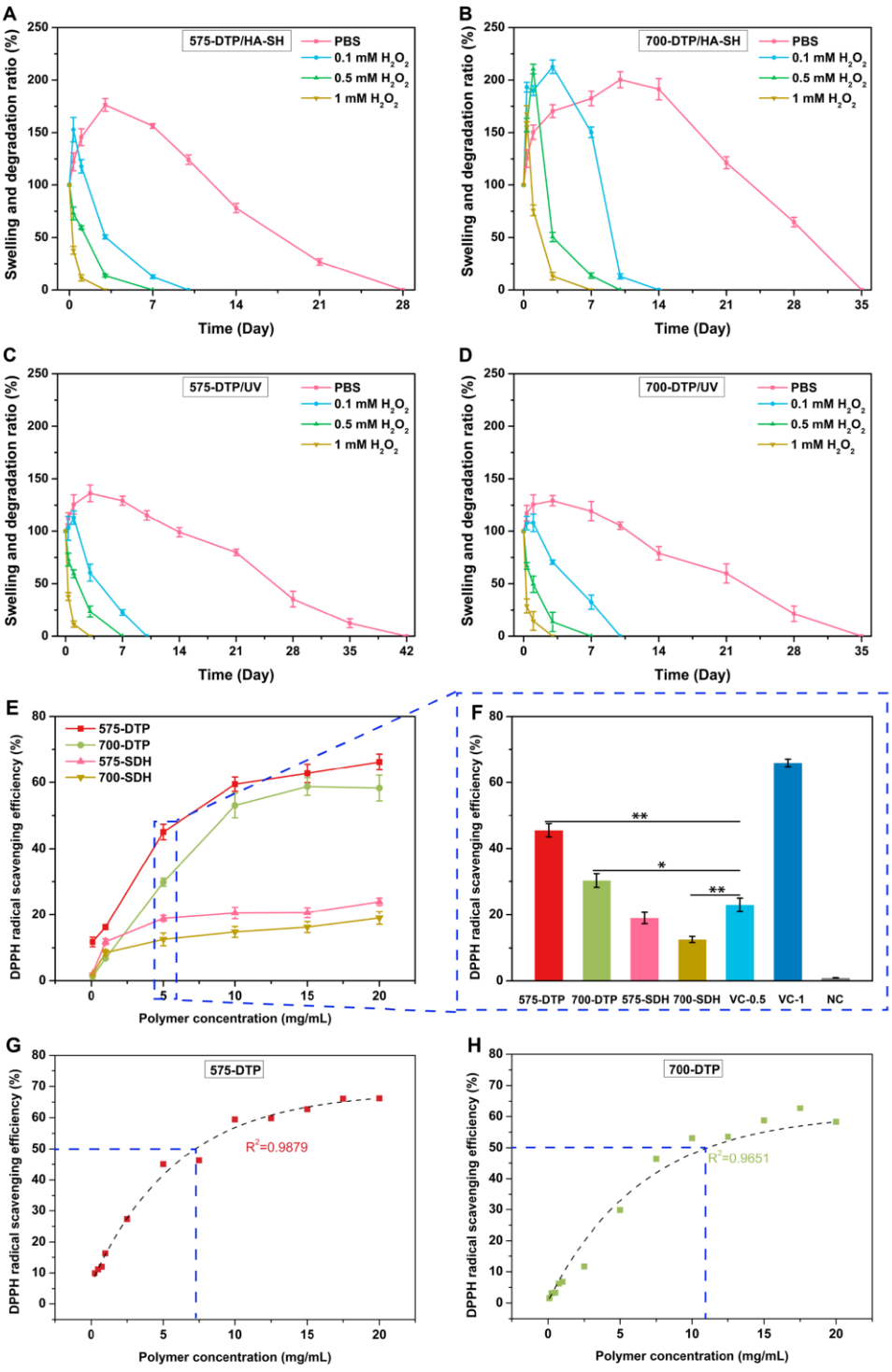


Figure 6. *Degradation rates of hyperbranched poly(β-hydrazide ester) macromers (HB-PBHE) -based hydrogels in PBS and different concentrations of H_2O_2 at 37 °C: (A) 575-DTP/HA-SH; (B) 700-DTP/HA-SH; (C) 575-DTP/UV; (D) 700-DTP/UV. DPPH free-radical scavenging assay: (E) DPPH radical scavenging efficiency of different concentrations of HB-PBHEs. (F) DPPH radical scavenging efficiency of HB-PBHEs at 5 mg/mL using VC (0.5 and 1 mM) as the positive control and ethyl alcohol as the negative control. (G,H) EC_{50} determination of DTP-based macromers. *, $p < 0.05$; **, $p < 0.01$. Reproduced with permission from ref. 10. Copyright (2018) American Chemical Society.*

4. ROS-producing therapeutic approaches

Injectable antioxidative hydrogels are promising options for therapies of many ROS-related disorders. Conversely, ROS are also utilised as weapons against cancers or infections due to their unique properties and activities involved in cell death. Due to the selective sensitivity of cancer cells to ROS, various drugs have been reported to induce the excessive generation of ROS to kill cancer cells[109]. However, they also increase the ROS level in healthy cells, which may induce DNA damage and cause secondary malignancies[109]. Furthermore, several conventional or novel therapeutic interventions, including nanotherapy, cold atmospheric plasma treatment, and photodynamic, sonodynamic and radiation therapies, have been demonstrated to combat tumour or infection via ROS overproduction. Comparably, many ROS-producing hydrogels have been reported for biomedical applications. As seen in the schematic illustration (Figure 7), the utilisation of injectable antioxidative hydrogels to protect surrounding healthy tissues, combined with ROS-producing therapy, is potentially a game-changer. The following introduction of those interventions aims to provide a comprehensive understanding of ROS-producing medical applications to accelerate the potential development of injectable antioxidative hydrogels in collaborating with those applications for optimal therapeutic outcomes and, more importantly, minimised side effects.

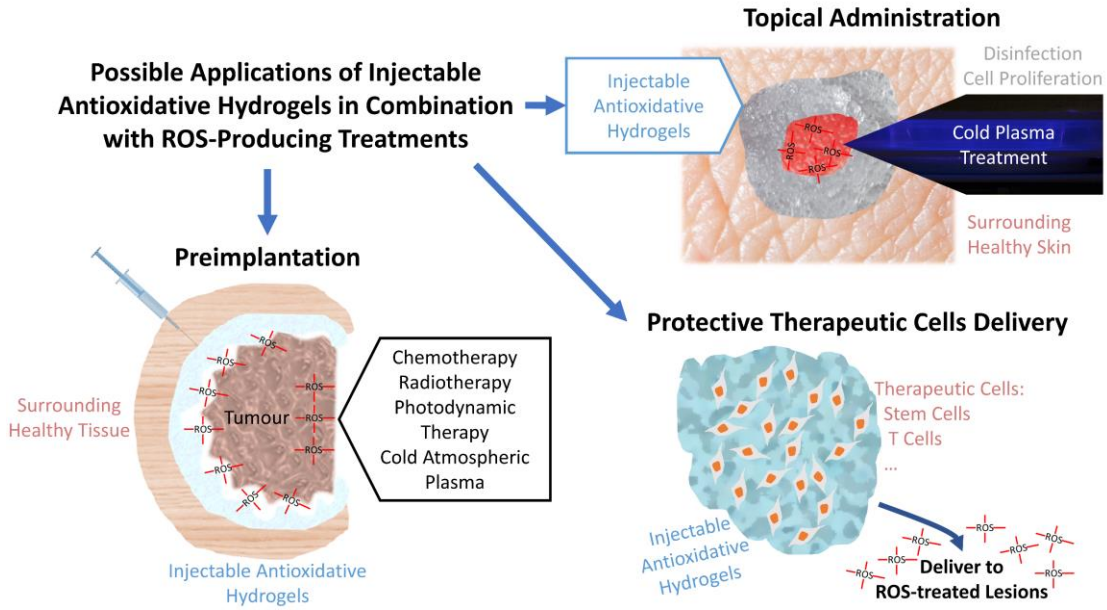


Figure 7. *Schematic illustration of potential application of injectable antioxidative hydrogels in combination with ROS-producing approaches for maximum efficacy and minimum adverse effects.*

4.1 ROS-producing injectable hydrogels

Due to the diversity in monomers, functional groups and cargos, various functional injectable hydrogels have been developed. Several ROS-producing hydrogels have been reported and presented promising application in biomedical areas, relying on their high biocompatibility and tuneable physicochemical properties. One of the majority applications of ROS in biomedicine is disinfection. Whereas the commonly used antibacterial and antiviral agents may lead to adverse effects and the potential generation of 'superbugs', strains of bacteria, viruses, parasites and fungi that are resistant to the majority of antibiotics and other common medications, the direct targeted delivery of ROS can efficiently damage the pathogens for excellent disinfection. Although we face challenges for precise and sufficient ROS delivery to treat infected areas, the development in ROS-triggering injective hydrogels can potentially overcome the obstacles.

In many research, ROS production by injectable hydrogels is achieved by entrapped or conjugated ROS-triggering nanomaterials[110–114]. For instance, an injectable hydrogel was synthesised by the rapid assembly of dopamine and folic acid crosslinked by transition metal ions[112]. The polydopamine in the hydrogel coated around carbon quantum dot-decorated ZnO (C/ZnO) NP can generate ROS and heat under 660 and 808 nm illumination, and thus presented significant antibacterial efficacy for potential applications in the reconstruction of bacteria-infected tissues (i.e. exposed wounds)[112]. Similarly, a functional porous polyvinyl alcohol hydrogel incorporated with ROS-producing CuS@MoS2 microspheres has been reported for excellent disinfection and improved wound healing efficacy[114]. Kumari *et al.* described a DNA based hybrid hydrogel, covalently conjugated with carbon dot and protoporphyrin IX, which has sustained photoinduced ROS-antimicrobial activity[115]. The carbon dot acted as crosslinkers and energy donors to excite protoporphyrin IX, the photosensitizer, to generate ROS[115]. On the other hand, ROS-producing injectable hydrogels can also be used to regulate cellular activity in tissue engineering by controlling the efficacy and rate of ROS production. For example, an injectable collagen hydrogel that is conjugated with biocompatible carbon dot NP through crosslinker genipin, is reported to generate a moderate amount of ROS in combination with photodynamic therapy[116]. The hydrogel-producing ROS was proven to accelerate cell proliferation and chondrogenic differentiation during cartilage regeneration[116].

Nonetheless, ROS production from injectable hydrogels can potentially cause oxidative stress to surrounding tissues, and the possible leakage of ROS-triggering nanomaterials or degradation of hydrogels can induce additional risks. Thus, due to the implantation feasibility of injectable hydrogels, the co-delivery and *in situ* co-formation of two hydrogels, using antioxidative hydrogels for protection or inhibition or excessive endogenous ROS and ROS-producing hydrogels targeting lesion or regulate regeneration, will potential provide optimal treatment for the complicated and delicate ROS homeostasis.

4.2 ROS-producing nanoparticles

In addition to being the cargos in injectable hydrogels, there have been extensively and in-depth investigation into the biomedical use of functional nanoparticles. Nanotechnology has been widely applied in the healthcare field due to the novel and unique physical and chemical properties of various nanomaterials. As a strong adjunct in pharmacology, NP are useful as imaging agents [117,118], drug carriers [119], radiosensitisers [120] and cancer therapeutics[121]. In addition to functioning as adjuncts, more specific types of NP were also found to have intrinsic cytotoxic properties, which can be useful for cancer treatment. The mechanism of NP-induced cytotoxicity can vary according to the material, size and other characteristics of the NP and further study is required. However, it has been shown that reactive species play a crucial role in the cytotoxicity induced by many types of NP, including silver[122,123], copper oxide[124,125], iron oxide[126], zinc oxide[127] NP and carbon nanotube[128,129], etc.

A majority of nanoparticles with cytotoxicity are metal-based, and can generate ROS at the NP surface and/or in solution, by release of metal ions from the NP surface. Meanwhile, the high levels of intracellular metal ions released by internalised NP also play a role in the toxicity. The release of toxic ions after the uptake of NP, which will lead to significant cellular changes, has been called the "Trojan-horse" mechanism[125,130].

For example, silver nanoparticles (AgNP) have emerged as a promising medical technique in recent years. AgNP have been shown to induce the generation of ROS on their surface in the cytoplasm, leading to intracellular oxidative stress, increased membrane permeability and inactivation of proteins and enzymes, and thus causing apoptosis or necrosis of tumour cells[131–133]. In addition, generation of ROS was elevated (>10 fold) and more GSH depletion was detected when cells were treated

with smaller, more reactive AgNP[122]. The IC_{50} value of 10 nm polyvinyl alcohol-coated AgNP against glioblastoma cells was determined to be ~4.30 μg/ml[134]. The EC_{50} of AgNP against alveolar macrophages decreased from >75 to ~27.9 μg/ml when the size changed from 55 nm to 15 nm. Furthermore, AgNP-55 nm presented no significant GSH depletion, but AgNP-15 nm depleted 100% GSH at 50 μg/ml and 67.8% GSH at 10 μg/ml after 24 h exposure in alveolar macrophages[122]. Copper-based nanoparticles have also long been known to generate ROS by Fenton-like and Haber-Weiss reactions, etc.[135]. Evidence showed that copper(II) oxide nanoparticles (NPCuO) can produce ROS on their surface or by releasing dissolved copper ions[136]. Electron paramagnetic resonance measurements determined that the hydroxyl radical is the main ROS generated by NPCuO, and DNA damaged by copper-generated ROS was also detected[125]. Javed *et al.* demonstrated that zinc oxide nanoparticles (ZnONP) can selectively kill cancer cells through ROS[127], and this study detected a significantly higher level of oxidant and lipid peroxidation in ZnONP-treated cells. Meanwhile, ZnONP was found to induce GSH depletion and decreased activity of several antioxidant enzymes in cancer cells, such as superoxide dismutase, catalase, GSH peroxidase and GSH reductase[127]. Pan *et al.* proposed that gold nanoparticles can trigger the formation of intracellular ROS from dioxygen, and that intracellular ROS can then cause mitochondrial permeability transition, which results in mitochondrial dysfunction and eventually in cell death by necrosis[137]. Although the promising anti-cancer effects of many nanomaterials have been reported, the possible adverse effects from leakage of reagents or ROS are a concern. Potentially, the adverse effects can be eliminated by injectable antioxidative hydrogels. Nanoparticles also have been used as photosensitisers and sonosensitisers in cancer treatment. As sensitisers, NP can be activated by local irradiation at a specific wavelength or ultrasound to generate ROS. The ROS generated in photodynamic therapy and sonodynamic therapy will be introduced in the following sections.

4.3 ROS-producing cold atmospheric plasma

Plasma, a form of ionised gas, is one of the four fundamental states of matter and accounts for most of the matter in the known Universe. Early biomedical applications of plasma were focused on the heat and high temperature of thermal plasma for the purposes of tissue removal, sterilisation, and cauterisation[138]. Technological advances have allowed researchers to generate cold atmospheric plasma (CAP), also known as

non-thermal atmospheric plasma (NTAP), which possesses ambient temperatures and approximately 1.0 atmospheric pressure. Even though cold plasma is generated by adding energy to a gas, releasing electrons from nuclei of atoms, electrons in CAP can be at several million K whereas the nucleus of atoms is at room temperature (thermodynamic disequilibrium state). The application of cold atmospheric plasma allows direct treatment of cells or live tissues with ionised gases without risking thermal injury.

Known biomedical applications of CAP include cancer therapy[38], sterilisation[139], wound healing[140], blood coagulation[141] and viral destruction[142]. CAP has also been investigated as a novel method to enhance cell transfection[143] and promote cell proliferation[144]. CAP generates a unique physical and chemical environment when exposed to biological tissues including activating short- and long-lived reactive nitrogen and oxygen species. Reactive nitrogen species (RNS) include peroxynitrite (ONOO-) and nitric oxide radicals (NO), while hydroxyl radicals (OH), oxygen atoms (O), and oxygen negative ions (O_2^-) are among the ROS in this environment. In addition, CAP generates photons as well as heat, pressure gradients, charged particles, and electrostatic and electromagnetic fields[145–147], many of which are known to induce biological effects. For example, peroxynitrite (ONOO-), which also occurs naturally[145], can initiate lipid peroxidation reactions and help against infection during inflammation[148], whereas ROS can cause DNA damage and induce apoptosis by activating the cell death receptors in the TNF/NGF-family [149]. These high fluxes of ROS also have significant effects in inactivating fungi, viruses and bacteria [138,150,151]. Although the US Food and Drug Administration (FDA) have approved CAP equipment, precise delivery and control of adverse effects are the critical challenges for successful application of CAP technologies.

4.4 ROS generated in photodynamic therapy

Photodynamic therapy (PDT), known as photochemotherapy of tumours, is an emerging, promising and FDA-approved technology used for the treatment of neoplasms. By the administration of light-sensitive photosensitisers (PS) and local irradiation with appropriate wavelengths, PDT is able to selectively eliminate cancer cells[152]. PS usually have no dark cytotoxicity until excited by irradiation with a certain wavelength and can be cleared rapidly by health tissues [153]. After excitation by light irradiation, PS are transferred from the ground state to the singlet state and later to

the triplet state[153]. The energy can then be transferred from triplet state PS to intracellular oxygen and induce ROS generation in cancer cells[154].

With an adequate supply of oxygen, PDT leads to direct cancer cell death, strong local inflammatory responses and microvascular damage[153]. The mechanisms of direct cell death include the activation of three pathways: apoptosis (predominantly), necrosis and autophagy, leading to cell death via ROS[154]. For instance, the ROS generated by mitochondria-associated PSs can inhibit the Bcl-2 family proteins located on the mitochondrial membrane, and lead to permeabilisation of the mitochondrial outer membrane and release of inter-mitochondrial membrane space (IMS) proteins, including caspase activators such as Smac/DIABLO, Omi/HtrA2, cytochrome c, as well as non-caspase apoptosis inducers such as apoptosis-inducing factor (AIF) and endonuclease G (EndoG)[155]. In addition, some PSs can accumulate in the vascular endothelial cells via specific receptors and diffusion[156–158]. Thus, the vasculatures of cancer cells also can concentrate PSs. When the tumour is exposed to appropriate irradiation, vascular walls will be disrupted by the PDT-induced oxidative stress, leading to a series of events, including stasis, leakage, collapse and blockage of the vasculatures[154,158].

4.5 ROS generated by therapeutic ultrasound

Sonodynamic therapy involves ultrasound-induced inertial cavitation which can produce ROS alone or together with sonosensitiser, and leads to a series of molecular reactions and finally to cell death[159]. The sonosensitisers, which can be excited by inertial cavitation and generate free radicals, have been developed extensively in recent years[160].

When ultrasound was transmitted into a focused area of tissue, the molecules inside cells will oscillate, and the average distance between the molecules may become greater, which can induce the formation of the cavity and draw gas/vapour out of solution to create bubbles[161]. Subsequent ultrasonic waves then can cause the oscillation of those bubbles, termed inertial cavitation or acoustic cavitation[162]. Bubbles will rapidly expand and suddenly collapse when exposed to higher-intensity ultrasound. The violent collapse can cause local energy release, shock waves, temperature rises and finally, generation of ROS at microscopic level[163,164]. The efficiency of ROS generation can be affected by the intensity, frequency and sequence of ultrasound pulses transmitted into the targeted tissue. It has been shown that

trigger high-intensity focused ultrasound, composed of medium-intensity sustaining burst and high-intensity short pulse, enhanced the generation of ROS, and that high-intensity pulse followed by low-intensity pulse also improved the rate of ROS generation[165,166]. Nishitaka *et al.* employed KI method to measure the ROS generated by ultrasound: I^- is oxidised to I_3^- by ROS, causing absorbance change at 355 nm, and 0.015 to 0.02 absorbance change was measured after and before ultrasound exposure[165]. Interestingly, on the other hand, by observing HeLa cells exposed to a 1.5-MHz ultrasound (13.33 μs duration and 0.70 MPa peak negative pressure) at the single-cell level, the intracellular ROS level was found to correlate with sonoporation: (i) ROS decreased rapidly along with extracellular diffusion of dichlorofluorescein due to membrane perforation and complete membrane resealing within ~120 s; (ii) ROS increased in reversibly sonoporated cells in the following 270 s; (iii) ROS level reduced to depletion in irreversibly sonoporated cells during this time interval[167].

Evidence also showed that sensitisers can be excited by the emission of light or extreme temporary heat occurring during acoustic cavitation. The energy is then released and transferred to intracellular oxygen when the sensitisers return to the ground state, thus generating ROS[168]. The addition of nanosensitiser was proven to have synergistic effects on ROS generation in combination with an ultrasound. For instance, it was shown that ultrasound treatments with hydrophilised titanium dioxide nanoparticles suppressed the growth of tumour more than 15-fold in a murine model, compared to the ultrasound-untreated group[169]. Sonodynamic treatment with other chemical sensitisers, such as hypocrellin B and protoporphyrin IX, has been proved to cause excessive accumulation of ROS, thereby inducing the death of cancer cells[170,171]. ZnO nanocrystals (NCs), functionalized with amino-propyl groups (ZnO-NH_2 NCs) was demonstrated to assist pulsed ultrasound efficiently generating ROS, detected by 5,5-dimethyl-L-pyrroline-N-oxide (DMPO) spin-trapping technique[172]. Using ultrasound at 1MHz and 1.5 W/cm^2 powder, up to 5 μM of DMPO-OH was detected in water with or without ZnO-NH_2 NCs, and with relative weaker ultrasounds (1 MHz, 0.9 and 1.2 W/cm^2), compared to pure water, 200 μg/ml of ZnO-NH_2 NCs enhanced the ROS generation 2 to >5 times[172].

4.6 ROS generation in radiation therapy

Radiation therapy (RT) is one of the primary treatments for neoplasms using high-energy ionising radiation (IR). RT is effective in controlling or killing various cancer cells

and has been prescribed for a large proportion of cancer patients as their sole treatment or combined with other interventions for more than a hundred years. The mechanisms of action of RT include the generation of free radicals or the direct deposition of energy by IR. Due to photoelectric effects and Compton effects, the energy track of IR is composed of electrons in matters, which induce ionisation and excitation[173]. In cells, the electrons interact with water and generate free radicals, including ROS and RNS[174]. The oxidative stress induced by ROS/RNS causes damaging lesions of cellular macromolecules, including DNA, protein and lipids[175]. Meanwhile, the cytotoxic effect is also related to the direct deposition of energy by IR, which is highly penetrating and able to cause irreparable damage to genetic material even at low doses[176]. However, due to the high cytotoxicity and non-targeted effects of radiation, the radiation must be accurately delivered to tumour tissue while sparing normal tissue to improve progression-free survival (PFS), overall survival (OS) and quality of life of patients[175].

Photodynamic, sonodynamic and particularly radiation therapies are widely applied in cancer treatment and other diseases. The development of diagnostic techniques and the refinement of those therapies have brought the accurate delivery of therapeutic ROS and other effects to new heights. However, severe adverse effects, especially with higher doses, are still the obstacle to effective and well-tolerated treatments. Therefore, a possible combination of ROS-producing interventions and injectable antioxidative hydrogels could restrict the effects of ROS to the target lesion and protect surrounding healthy tissue, offering a promising solution to address the abovementioned challenges.

5. Concluding remarks: elimination or confine of ROS by injectable antioxidative hydrogels.

As the understanding deepens, the vital roles of ROS in physiological and pathological conditions become targets and weapons against diseases and for promoting health. Many studies demonstrating the mechanisms of ROS as secondary messengers in inflammation or cellular response to oxidative stress and damage are contributing to the treatment of ROS-imbalanced diseases. Additionally, due to the highly reactive and unique natures of ROS, they are optimal weapons against pathogens and present significant selective cytotoxicity to cancer cells; they have therefore already been applied or found to be important in many anti-tumour and anti-infection interventions.

Injectable antioxidative hydrogels have emerged as one of the most promising biomaterials for ROS-related biomedical applications. However, despite a large number of injectable or non-injectable antioxidative hydrogels that have been described in the field of biomedical research, especially in chronic wound healing, cardiovascular diseases and neurodegenerations, the clinical applications of injectable antioxidative hydrogels are still limited. Several fundamental critical points need to be considered, including 1) biological safety, stability and degradability, 2) appropriate physicochemical properties, 3) sufficient controllability for the redox balance 4) feasibility of the fabrication and in situ gelation. Injectable antioxidative hydrogels that meet the above conditions are likely to contribute to tremendous leaps and breakthroughs for the therapy of many ROS-triggered diseases.

On the other hand, as told from the famous Chinese folklore, whereas ROS scavengers are like the best shield, ROS are also used as one of our best spears against diseases, such as cancer and infections. There is no need to create a conflict by considering ROS-generation interventions and injectable antioxidative hydrogels as being incompatible. Due to the high moldability and the minimally invasive *in vivo* delivery of the hydrogels, they can potentially be used as robust protectants for the normal tissue surrounding the lesions targeted by ROS-producing interventions, such as radiotherapy, ROS-inducing chemotherapy, hyperthermia and sonodynamic therapy. These major treatments generating exogenous ROS can then achieve maximal efficacy with minimal side effects when used in combination with injectable antioxidative hydrogels, giving the development of injectable antioxidative hydrogels further promising possibilities.

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Conflict of Interest

The authors declare no conflict of interest.

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