Modifying superoxide dismutase for improved biopharmaceutical properties

REVIEW

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ABSTRACT

The superoxide dismutase enzyme (SOD) contributes to the physiological equilibrium between pro-oxidants and antioxidants, by destroying the superoxide anion (O_2^-). However, its therapeutic use is limited by its fast clearance from the bloodstream and inactivation by its own reaction product, *i.e.* hydrogen peroxide. Here is a summary of the main strategies developed to circumvent these limitations. The therapeutic fitness of SOD could be achieved by chemical modification or by using polymeric hydrogels for its controlled release. SOD can be chemically modified with other macromolecules like carboxymethyl cellulose (CMC). Otherwise, it could be encapsulated in liposomes or absorbed in CMC hydrogels. All these strategies increase the SOD half-life in the bloodstream, also improving its pharmacological properties.

Key words: superoxide dismutase, modified enzyme, pharmacological properties

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RESUMEN

Modificación de la Superóxido dismutasa para mejorar sus propiedades biofarmacéuticas. La superóxido dismutasa (SOD) destruye el anión superóxido ($O_2^{\cdot-}$) lo que contribuye al mantenimiento del equilibrio fisiológico antioxidante-prooxidante. Sin embargo, debido a su rápida eliminación de la circulación sanguínea y a su inactivación, como resultado de la interacción con el propio producto (H_2O_2) de la reacción que ella cataliza, su uso terapéutico está limitado. La aplicación terapéutica de la SOD podría aumentarse por modificación química o mediante el uso de hidrogeles a base de polímeros para la liberación controlada de SOD. En este artículo se reportan varias estrategias que se han desarrollado para resolver estas dificultades, incluyendo la encapsulación de la proteína en liposomas, así como la modificación química de enzimas por otras macromoléculas, sobre todo la modificación química de la SOD por carboximetilcelulosa (CMC) y la absorción en hidrogeles de CMC. Estas transformaciones incrementaron el tiempo de vida media de la SOD en la circulación sanguínea para esta enzima y mejoraron sus propiedades farmacológicas.

Palabras claves: superóxido dismutasa, enzima modificada, propiedades biofarmacéuticas

*I*ntroduction

One of the leading research areas in medical sciences comprises the development and application of antioxidant agents for diminishing injuries caused by free radicals [1]. Free radicals are generated from molecular oxygen, starting with the overproduction of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) radicals that are subsequently converted into potent oxidants like hydroxyl, hypochloric acid and peroxynitrite [2]. When the level of these free radicals surpasses the various antioxidant barriers of the organism, chemical lesions tend to accumulate and the damage of biological structures become evident in a process known as oxidative stress [3]. It is characterized by unbalanced antioxidant defenses against a rising production of the aforementioned free radicals [3], and commonly involved in different pathological conditions like hypertension, thrombosis, diabetes, reperfusion ischemia, acute respiratory distress syndrome, pulmonary edema, acute pancreatitis, inflammation, mutagenesis, carcinogenesis, aging and neurological disorders [4, 5]. Some of them (*e.g.* cancer, diabetes) are among the main causes of death in developed countries, while other medical entities like arthritis, nephropathies, dementia and aging are accelerated by the magnitude of the oxidative stress [6]. This evidences the need of efficient antioxidant defenses for preserving health.

Under certain conditions (*e.g.* ischemia and inflammation) free radicals can be generated so quickly that they can outreach the neutralizing capacity of superoxide dismutase (SOD) and catalase enzymes, supporting the subsequent chemical reduction of the superoxide radical into other farther reactive free radicals like hydroxyl. These highlight the importance of neutralizing the superoxide radical. Therefore, an effective therapeutic strategy for ameliorating the damage caused by oxidative stress could involve interfering with and degrading (detoxifying from) O_2^- and H_2O_2 before reaching a burden [7].

Among the enzymes mediating these processes, SOD (converting O_2^- into H_2O_2) and catalase (converting H_2O_2 into water) have been considered potential antioxidant drugs. So far, several animal studies and human clinical trials have suggested that SOD and catalase confer a weak protection against vascular oxidative stress [8], with the use of the superoxide dismutase enzyme (SOD,

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EC 1.15.1.1) as the most promising. However, SOD applications for these purposes are limited by its very short half-life in the bloodstream (approximately 5 minutes), demanding repeated administrations for achieving the therapeutic effect [9]. Additionally, the SOD becomes inactivated by its own reaction product, hydrogen peroxide, also generating very toxic radical species in the organism [10].

Two main approaches have been attempted to circumvent these problems with the clinical application of SOD. One comprises improving SOD properties by chemical modification through covalent linkage to hydrophilic molecules [11, 12]. Biocatalysts modified by these means have been parenterally administered as long-term effect pharmaceuticals in animals and humans [13]. That is why, a great number of natural and synthetic polymers have been used as agents for modifying the antioxidant enzymes [14-16].

Alternatively, the therapeutic effectiveness of enzymes can be increased by using controlled release systems composed of hydrophilic polymer hydrogels of proven biocompatibility [17-19]. These biomaterials can also promote the adhesion and proliferation of cells involved in tissue repair and regeneration [20, 21].

Superoxide dismutase: medical relevance

Superoxide dismutases (SOD, superoxide-oxidoreductases, 1.15.1.1.) comprise a group of metaloenzymes frecuently found in aerobic, aerotolerant and some obligatory anaerobic organisms; they are essential for protecting from the toxicity produced by partially reduced metabolites, which are generated during the normal biological reduction of molecular oxygen.

The SOD destroys the superoxide radicals, contributing to a physiological balance between pro-oxidants and anti-oxidants [1]. In its pharmaceutical form, Orgotein, the SOD is a potent anti-inflammatory agent. The best characterized one is the SOD extracted from bovine erythrocytes, composed by two catalytic subunits, bearing one Cu^{+2} and one Zn^{+2} atoms each (CuZn-SOD)[22, 23].

SOD is relevant for medical purposes due to its therapeutic potential in oxidative stress-related diseases and in alleviating their related symptoms [24]. There are three forms of SOD according to the associated metal cofactor: CuZn-SOD, Mn-SOD and Fe-SOD. They are unrelated according to sequence and tertiary structure analyses, indicating independent evolutionary outcomes to the common selective pressure of oxygen metabolism and its associated toxicity. The Fe-SOD is normally found in prokaryotes [25]. In eukaryotic cells, three forms of SOD are found according to their location: mitochondrial Mn-SOD; cytosolic CuZn-SOD; extracellular CuZn-SOD [24]. All these enzymes to destroy the superoxide radical before generating other radical species or reacting with susceptible biological molecules [26].

All of them catalyse the conversion of superoxide into hydrogen peroxide:

$$2\ O_2^{\cdot-} + 2\ H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2$$

Since $O_2^{\cdot-}$ concentrations are normally low, the reaction depends on diffusion; however, the association of the enzyme with its substrate is not limited to simple diffusion and interaction.

In spite of the encouraging results as an anti-inflammatory agent in pre-clinical and clinical studies [9, 16], the use of the native SOD enzyme is limited by its short half-life and its sensitivity to inactivation by its own reaction product [9]. Therefore new formulations are demanded for improving its pharmacokinetic properties.

SOD formulations. Polymer-modified SOD

The use of protein-based pharmaceuticals has been extended in recent years, including enzymes, hormones, monoclonal antibodies, epidermal growth factor [27], and others. Nevertheless, their increased metabolic turnover, antigenicity, immunogenicity, and low physiological stability have limited the systemic therapeutic administration of these products [9].

Several methods were developed for the physical entrapment of such bioactive substances in natural or artificial structures (*e.g.* liposomes, microspheres, erythrocytes) to circumvent the above-mentioned limitations [27-29]. Another effective strategy for alleviating those problems involves the chemical modification of the protein's surface by covalent linkage to soluble, non-toxic polymers [30-32]. This procedure improved the pharmacological, pharmacokinetic and immunological properties of therapeutically relevant proteins like peroxidases [14], catalase [15], SOD and others [16, 33].

The polymers established for the chemical modification of proteins include: polyethylen glycol (PEG, the founder [34-36]), dextran, polyvinyl pyrrolidone, hyaluronic acid (HA) and others [37-39].

Beckman and co-workers chemically modified catalase and SOD with PEG in 1988 [40]. They reported that after adding the modified enzymes to a cell culture, cells increased resistance to oxidant effects of reactive oxygen species (ROS), the modification mediated the attachment of the enzyme with the cellular membrane and the entry into the cell. This was corroborated by an increased enzyme activity inside cells after incubating with the enzyme conjugates. SOD conjugated to PEG also showed improved anti-inflammatory properties and an increased half-life in blood [34, 41]. This last parameter is probably related to a delayed renal filtration of the protein, resulting from a quenched ionic charge of the chemically modified amino groups of the protein after their conversion into amide groups [42]. The increase of molecular weight of the enzyme after conjugation also correlates with augmented half-life in the blood after systemic administration [42-43], contributing to its increased anti-inflammatory activity.

The impact of the administration route on the pharmacokinetic and pharmacodynamic properties of PEG-conjugated proteins (for the SOD also [44]), their antigenicity and immunogenicity, has been studied *in vitro* and *in vivo*. A decreased lipid peroxidation in blood vessels has been documented after administering

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modified SOD. The PEG-SOD was as effective as the native SOD for treating reperfusion arrhythmia and myocardial ischemia. It was also effective in lungs, diminishing oxygen toxicity and lesions caused by *Escherichia coli* and it attenuated the reperfusion damage in renal and hepatic ischemia [13].

Another polyanionic conjugate of SOD to divynil ether and maleic anhydride known as DIVEMA showed effective SOD activity [45], with prolonged half-life and better attachment to hepatic receptors than the native SOD. As a result, a strong inhibition of the hepatic production of ROS in rats was attained, with the subsequent decrease in hepatic inflammation, compared to the discrete effect obtained with the unmodified enzyme [46].

In rats with pulmonary edema caused by bronchial reperfusion after a three-day ligation, this polyanionic conjugate effectively protected against progressive edema, an effect absent in animals treated with native SOD or DIVEMA alone. Results were confirmed by electron microscopy analyses. SOD-DIVEMA-treated animals also showed lower leukocyte infiltration at the vascular endothelium, unlike animals treated with SOD alone [47]. This indicated an anti-inflammatory effect at the initial steps of leukocyte adhesion and cellular expansion.

SOD has been additionally modified with HA (disodium salt). The enzyme was coupled through its amino groups to the hyaluronate carboxyl groups, by reacting with 1-ethyl-3-(3-dimethyl aminopropyl) carbodimide. The resulting SOD-HA conjugate retained a 70% activity of the unmodified SOD. The conjugate was essentially non-immunogenic in mice, and exhibited much higher anti-inflammatory activity than HA or native SOD in models of inflammatory diseases such as foot-pad ischemic edema in mice, carageen-induced pleurisy and adjuvant arthritis in rats [39].

In burned tissues, the above mentioned inflammatory signs are notorious, with overproduced biochemical mediators and activated leukocyte and endothelial cells influencing local and distal sites [48].

The recombinant CuZn-SOD (rCuZn-SOD) has been applied in ischemic tissues damaged by ROS and in burned animals (*i.e.* rabbits with scalded backs). The efficacy of liposomal oxygen free radical scavengers like rCuZn-SOD in burn wound healing evidenced their relevance for reducing tissue damage [49]. rCuZn-SOD-treated rabbits exhibited reduced edema formation, smaller wounds and tissue necrosis than control animals, with significantly faster re-epithelialization after 3 weeks, and diminishing inflammation [51]. These effects could be related to the role of SOD in scavenging the superoxide anion, protecting membrane phospholipids from peroxidation and, therefore, cellular permeability that generates skin inflammation [51].

Another ROS related disease, rheumatoid arthritis, is characterized by an autoimmune reaction against the joints by infiltrated, ROS-producing blood-derived cells that promote oxidative stress [52]. One approach to counteract this process comprises the use of antioxidants as therapeutic agents. For example, the free radical scavenger enzyme SOD may be used as a therapeutic agent in rheumatoid arthritis, but it is limited by its rapid elimination from the blood [53]. Some studies have demonstrated the use of PEGylated liposomes (PEG-liposomes) for targeting SOD to arthritic sites, evidencing that SOD can be targeted to inflammation sites most efficiently via small-sized PEG-liposomes [52]. On the other hand, bovine CuZn-SOD injections have acted through immunomodulatory networks in rats affected by adjuvant-induced polyarthritis [53].

The SOD has also been encapsulated into mucoadhesive chitosan-coated liposomes prepared using soybean lecithin, stearylamine, phosphatidyl glycerol and cholesterol. Stability tests for these SOD-loaded liposomes showed no significant loss of enzyme activity within 1 month at 4 ºC or within 2 days at 37 ºC. Indeed, these chitosan-coated SOD-loaded vesicles could be very useful as carriers and delivery vehicles for SOD and drugs targeting the mucosal tissues [29].

Based on the concept that removal of superoxide modulates the course of inflammation, synthetic, low-molecular-weight mimetics of the superoxide dismutase enzymes have been designed as therapeutics for several unrelated diseases [54].

Another approach for increasing the therapeutic application of proteins and peptides involves the use of controlled release systems made up of hydrogels [55], but we have no current knowledge on the use of this kind of formulation for delivering SOD *in vivo*.

*H*ydrogels and their applications

The variety of materials employed for making medical devices (metals, ceramics, polymers, and biomaterials), have recently included gels [56]. They are particular states of the matter between liquids and solids, composed of a polymeric and reticulated solid matrix permeable to water [57, 58]. They are technically referred to as semisolid systems of small solid portions dispersed in relatively high amounts of liquids.

These biomaterials are commonly used as supports for cellular proliferation, providing a tri-dimensional network for tissue formation and maintaining its structure and function [59, 60]. Due to the intertwining of their constituting hydrophilic polymers, hydrogels are capable of absorbing huge amounts of water (more than 20% of their weight) or biological fluids and swell while maintaining their tri-dimensional structure [61]. They were first applied more than thirty years ago, when Wichterle and Lim [62] suggested in 1960 their use in medicine, due to their physical resemblance to cellular matrixes. They proposed the use of a hydrophilic network of 2-hydroxyethyl metha-crylate (PHEMA) for making contact lenses, an application still available today [20]. Since then, the use of hydrogels was extended to other biomedical [62] and pharmaceutical [64] purposes; they are applied as extracellular matrixes for tissue engineering [65], skin grafting (in burns) [66] and also for drug controlled release [67], among others.

Several procedures for the synthesis and characterization of hydrogels for medical and pharmaceutical applications have been described in the scientific literature, starting from natural, reticulated polysaccharides [60, 68].

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Some hydrogels generated by cross-linking hydrophilic polymers represent a relevant group of biomaterials in biotechnology and related sciences. Most of them show appropriated biocompatibility [69], causing very mild manifestations of thrombosis, inflammatory response and other lesions following administration. This is based on their reticulated structure and hydrophilic composition that allows an increased permeability to oxygen, nutrients and other soluble metabolites [70].

These biomaterials have changed drug release systems in recent years, increasing the therapeutic benefit of drugs administered by these means [64].

Polymers from natural, synthetic or semisynthetic sources, and bearing hydroxyl, amino, amide, ether, carboxyl and sulphonate groups as chain functional residues, are generally employed for generating hydrogels. Most of these hydrophilic hydrogels are composed of natural polysaccharides, such as hyaluronate, alginate and CMC [71]. They normally absorb a huge amount of water while preserving their integrity and elasticity.

Hyaluronan (Hyal) has been modified *in vitro* by inserting sulphate to hydroxyl groups. When primary ovine chondrocytes and endothelial cells in culture were incubated with this sulphated hydrogels, good adhesion and spreading responses were observed. These results suggested that sulphated Hyal containing materials could be used as biomaterials to aid cartilage repair and vessel endothelisation [17], although expensive for extensive application.

Several sodium alginate matrixes were compared in a recent study for producing bone grafts in reconstructive surgery [72].

Carboxymethyl cellulose (CMC), a low-cost commercial soluble and polyanionic polysaccharide derivative of cellulose, has also been employed in medicine [73], as an emulsifying agent in pharmaceuticals, and in cosmetics and the food industry. In biomedicine it has been employed for preventing post-surgical soft tissue and epidural scar adhesions [74]. It can also be used for the therapeutic application of the SOD, presented as hydrogels of CMC carrying the enzyme for its controlled release [73, 75]. There is a fifteen-year experience in breast surgery with CMC-based gel implants, with an increasingly reliable profile of very low toxicity and sustained viscoelas-ticity in grafts [73]. Valeriani *et al.* reported a similar experience [75].

Sanino and co-workers have proposed the use of CMC and hydroxyethyl cellulose-based gels as water absorbents in treating edemas [74], an alternative to current diuretic therapies for treating health conditions conducing to edema.

Our group developed methods for synthesizing SOD conjugates by chemical modification with carboxymethyl chitin [33], mannan [77] and CMC [78], respectively. CMC glycosylation was carried out by two methods: reductive alkylation with polyalhehydic CMC, periodate-oxidized polymer, and the formation of amide linkages through a carbodiimide catalyzed reaction [79]. CMC-based gels were obtained with varying cross-linking degrees (54% and 91%) by stoichiometrically adding 2-chloro-1-methylpiridin iodide (CMP-J), according to Magnani *et al.*[57]. The SOD enzyme was adsorbed into the hydrogel for its controlled release, rendering two formulations: SOD-CMC conjugates and SOD-CMC hydrogels [78]. Both formulations were chemically and biologically characterized, resulting in 1.2-1.8 moles of polymer per mole of protein (modified SOD) with acceptable SOD specific enzyme activity values (assessed by the xanthine oxidase method) [78].They overcame some of the limitations associated with the use of the native enzyme, promoting its clinical application [79, 80]. Moreover, up to 50% of the SOD was released from the SOD-CMC hydrogel after 72 h, indicating a controlled release kinetic [81]. The effects of these SOD-CMC conjugates and SOD-CMC hydrogels on the growth of *in vitro* cultured human fibroblasts were evaluated, and expressed by the cell proliferation inhibition index (CPII) as a biocompatibility marker, and cell morphology parameters (area and perimeter) were studied by electron microscopy. Results indicated a decreased CPII for SOD in both types of formulations (enzyme conjugates and hydrogels), with the lowest CPII for the 54% crosslinked CMC hydrogel (CPII=4.4±2.4) [78].

Considering the role of endothelial cells and fibroblasts on repairing and regenerating tissues injured by several processes (including oxidative stress), proliferation kinetics were also studied [82]. The added 54% and 91% crosslinked SOD-CMC hydrogels did not alter cell proliferation; they also significantly accelerated growth of cells incubated with the 54% crosslinked hydrogel. These results could be explained by gel morphology, being the 54% crosslinked hydrogel morphologically the most porous and permeable support for growing fibroblasts (as demonstrated by electron microscopy, see Figure 1a). These properties allow a better diffusion of metabolites, nutrients and gases for growing cells

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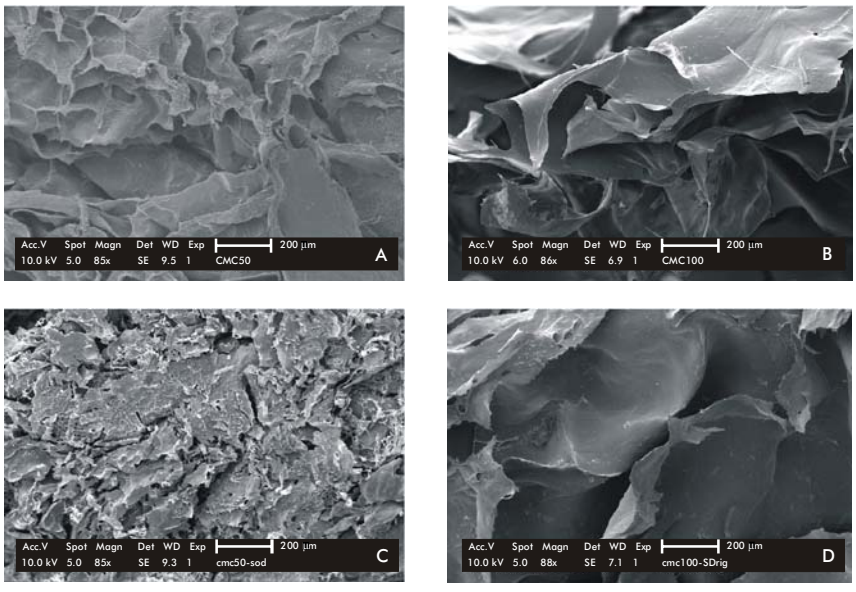

Figure 1: Scanning electron microscopy micrographs of 54 % and 91 % of cross-linking carboxymethyl cellulose hydrogels, alone (a and b, respectively), or carrying absorbed superoxide dismutase (c and d, respectively). See reference 78 for further details.

than for the case of the more compact 91% crosslinked hydrogel (Figure 1b and 1d). The size of the gel pore is also relevant for cell extension and vascularization after grafting [83].

All this evidenced that by absorbing the SOD into CMC hydrogels, the SOD can be effectively delivered for therapeutic purposes to tissues and organs damaged by free radicals, thereby increasing cell proliferation and healing. They also circumvent the known limitations of the native SOD without affecting its biocompatibility, significantly protecting SOD from inactivation by hydrogen superoxide, an effect unattained by other polymer modifications. These results obtained with CMC polymers are encouraging for future clinical trials, and would serve as methodologies for studying SODs isolated from other natural sources (*e.g.* plants) or to modify other clinically relevant proteins.

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