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Immobilizing Cu,Zn-Superoxide Dismutase in Hydrogels of Carboxymethylcellulose Improves Its Stability and Wound Healing Properties

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Abstract—Hydrogels of carboxymethylcellulose (CMC) with 50 and 90% cross-linking degree (CMC50% and CMC90%, respectively) were prepared and loaded with bovine erythrocyte Cu,Zn-superoxide dismutase (SOD) to obtain two drug delivery systems: SOD–CMC50% and SOD–CMC90%. Resistance of native SOD to inactivation by H_2O_2 and the effect of applying SOD–CMC hydrogels to open wounds of rats' back skin were examined and compared to that of SOD trapped into CMC50% and CMC90% hydrogels. Also, the effect of CMC50% and SOD–CMC90% on human fibroblasts proliferation was evaluated at different times. It was found that SOD in the hydrogel was more resistant to H_2O_2 inactivation than the native enzyme and at the same time it reduced the time necessary for wound healing. Furthermore, the highest cell proliferation value was found for the CMC50% hydrogels, which had a three-dimensional structure suitable for gas and nutrient exchanges and improving cell life conditions.

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Superoxide dismutase (SOD), a scavenger enzyme, catalyzes the dismutation of superoxide anion ($O_2^{\cdot-}$) into peroxide and molecular oxygen and thus protects cells, tissues, and biomacromolecules from free radical damage [1]. Considering the important role of $O_2^{\cdot-}$ in inflammation [2], infections diseases [3], ischemic disorders [4], tumor promotions [5], and aging [6], SOD plays a key role in the maintenance of physiological antioxidant–prooxidant balance [7]. Despite its importance as an anti-inflammatory agent, its therapeutic use is currently limited because of its short blood-life [8] and its inactivation by H_2O_2, one of its own reaction products [9].

The therapeutic application of SOD could be increased by conjugating it with polysaccharides or immobilizing it into hydrogels that can enhance its functional and structural stability and extend its half-life in blood [10-12]. In fact, hydrogels are three-dimensional networks of hydrophilic polymers that can adsorb substantial amounts of water [13]; they are generally characterized by good biocompatibility [14] and can promote cell adhesion [15].

Many polysaccharides have been used for pharmaceuticals and medical applications in the form of hydrogels [14-17]. Among them, great interest has been devoted to carboxymethylcellulose (CMC) because of its commercial low cost and biocompatibility [18]. In this work hydrogels of CMC with 50 and 90% cross-linking degree were prepared [19]. Then they were kept in contact with SOD to obtain SOD–CMC hydrogel systems. The inactivation of SOD by H_2O_2, repair of open wounds in the back skin of rats, and the effect of SOD–CMC hydrogels on *in vitro* cellular proliferation of human fibroblasts were further evaluated.

Abbreviations: CMC) carboxymethylcellulose; CMPI) 2-chloro-1-methylpyridinium iodide; DMEM) Dulbecco's modified Eagle's medium; DMF) N,N′-dimethylformamide; EDAC) 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride; FCS) fetal calf serum; SOD) bovine erythrocytes Cu,Zn-superoxide dismutase; TBA) tetrabutylammonium hydroxide.

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MATERIALS AND METHODS

Materials. Sodium salt of CMC (carboxymethylation degree 0.9 ± 0.1 per monosaccharide unit, molecular weight 100 kD) was supplied by Hercules Italia S.p.A. (Italy). Dimethylformamide (DMF), 2-chloro-1-methylpyridinium iodide (CMPI), formaldehyde solution, tetrabutylammonium hydroxide (TBA), 1,3-diaminopropane, xanthine sodium salt, xanthine oxidase, and resin Dowex 50WX8 were purchased from Fluka (Switzerland). Triethylamine and 1-ethyl-3(3-dimethylaminopropyl)carbodiimide hydrochloride (EDAC) were purchased from Merck (Germany). SOD (EC 1.15.1.1, 3500 U/mg), trypsin-EDTA (1×), Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), and L-glutamine–penicillin–streptomycin solution (200 mM L-glutamine, 10,000 U penicillin, and 10 mg streptomycin in 0.9% NaCl) were purchased from Sigma (USA). DMEM was supplemented with 10% FCS and 1% glutamine–penicillin–streptomycin solution: this medium is further referred to as DMEM complete medium.

Synthesis of SOD–CMC hydrogels. The CMC hydrogels were synthesized as previously described [19]. First, the sodium salt of CMC was converted into the TBA salt of CMC, soluble in DMF. Second, a stoichiometric amount of CMPI was added to the solution to activate about 50 or 100% of the carboxylate groups. Reticulation of the hydrogels was obtained using the 1,3-diaminopropane as the cross-linking agent, together with a small amount of triethylamine as a catalyst, with stirring for 3-4 h under a nitrogen flow at 4°C. Then the hydrogels were dried by lyophilization. Potentiometric titration was performed on the swollen hydrogel to measure the cross-linking degree [19]. Afterwards, 10 mg of each hydrogel was immersed in 1 ml of solution of SOD (1 mg/ml) in H_2O for 72 h at 4°C, time enough for hydrogels to swell completely and to absorb all SOD protein solution. Then the SOD–CMC hydrogels were freeze-dried after previous washing.

Resistance to inactivation by H_2O_2. Native enzyme and SOD–CMC hydrogel systems (equivalent to 1 mg SOD, 3500 U) were incubated at 37°C in 100 mM H_2O_2 and 20 mM sodium phosphate buffer, pH 7.0. Aliquots were removed at scheduled times, treated with 1 μg of catalase, and further assayed for SOD activity by the xanthine oxidase/nitroblue tetrazolium method [20].

Cell proliferation test. Four different samples, CMC50%, SOD–CMC50%, CMC90%, and SOD–CMC90%, were tested. Primary human skin fibroblasts were used to carry out the experiment. They were routinely cultured in DMEM complete medium. The cells were harvested using trypsin–EDTA (1×) and resuspended in fresh DMEM complete medium. Then they were pipetted into each well of a 24-well plate onto 10 mg of each sample (containing 1 mg SOD in the systems with enzyme), which was previously placed into the well. Finally the cells were incubated at 37°C under air containing 5% CO_2: the seeding density was of $5 \cdot 10^3$ cells/cm^2.

All samples were set up in triplicate and both cell growth and morphology were evaluated by daily inspection with an Olympus BX 40 light microscope. Cell count was performed by light microscope observation at 12, 24, 48, 72, and 96 h of cultivation, and the growth curve was plotted.

Wound repair test. Male Wistar rats of approximate weight 250 g were used. The animals were distributed at random in six groups of six animals each. A 2-cm-diameter wound was made in every animal's back skin, and a topical treatment of SOD, SOD–CMC50%, SOD–CMC90%, CMC50%, CMC90%, and the control was applied on the six different groups of rats every 72 h during the first week after wounding, as follows:

– group I, animals treated with 1 ml of solution of SOD (1 mg/ml);

– group II, animals were treated with 10 mg of SOD–CMC50% (containing 1 mg of SOD);

– group III, animals were treated with 10 mg of SOD–CMC90% (containing 1 mg of SOD);

– group IV, animals were treated with 10 mg of CMC50% (without SOD);

– group V, animals were treated with 10 mg of CMC90% (without SOD);

– group VI, animals were treated with 200 ml of phosphate buffer, pH 7 (control).

The wounds were observed daily during three weeks, time enough to observe the closing of the wounds [21].

Statistical analysis. For all statistical analyses, Microcal Origin 7.0 (Origin Lab Corporation) was used. The data were analyzed by ANOVA, and means were compared using Student's *t*-test. Differences were considered to be significant at $p < 0.05$.

RESULTS AND DISCUSSION

Resistance to inactivation by H_2O_2. The real cross-linking degrees (i.e. number of COO^- present in the polysaccharide chain involved in the cross-linking reaction) of the prepared CMC-based hydrogels determined by potentiometric titration were 50 and 90%.

As determined by the xanthine oxidase/nitroblue tetrazolium method [20], SOD interacting with CMC-based hydrogels was more resistant to H_2O_2-inactivation than the native enzyme. As can be easily seen in Fig. 1, native SOD was rapidly inactivated by H_2O_2 treatment with second-order kinetics and a half-lifetime of 20 min. However, after 4 h of incubation SOD–CMC50% completely retained the initial activity, whereas the SOD–CMC90% retained 77% of it.

These data are in complete agreement with previous reports about the ability of SOD conjugated with anionic polymers to maintain high catalytic activity after incuba-

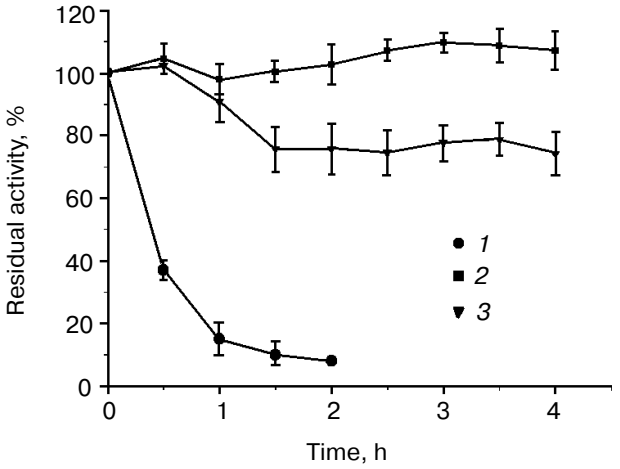

Fig. 1. Kinetics of inactivation of native (*1*), SOD–CMC50% (*2*), and SOD–CMC90% (*3*) SOD preparations against 100 mM H_2O_2. Data from representative experiments performed in triplicate (see "Materials and Methods" section).

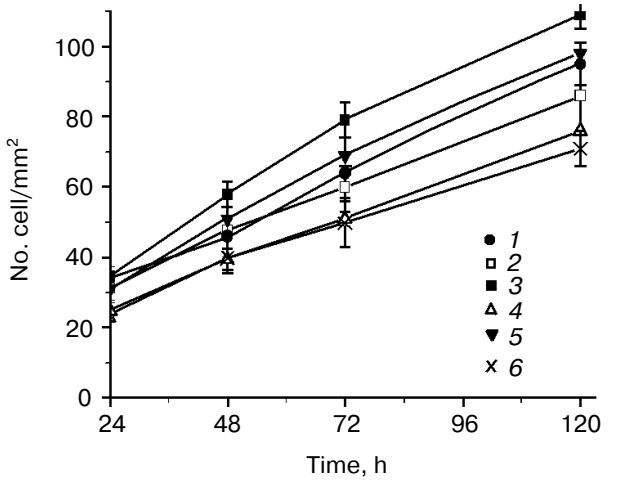

Fig. 2. Growth of human fibroblasts in the presence of SOD (*1*), CMC50% (*2*), SOD–CMC50% (*3*), CMC90% (*4*), SOD–CMC90% (*5*), and control (*6*). Data from representative experiments performed in triplicate (see "Materials and Methods" section).

tion with H_2O_2. This functional and structural stabilization was strictly correlated to chelating properties of the modifying polymers [22, 23]. Also in this case we may suppose that the three-dimensional structure of the CMC hydrogels protected the enzyme from the chemical attach of H_2O_2. Furthermore, the free carboxyl groups present along the CMC chains at pH 7.0 have negative charge, so they may capture free Cu^{2+} present in the SOD structure and consequently prevent possible side reactions (i.e., the Fenton reaction).

Test of cell proliferation. According to the data reported in Fig. 2, the primary human fibroblast proliferation in contact with CMC50% and CMC90% is significantly lower than that with native SOD and SOD–CMC hydrogels. In particular, CMC50% showed cell proliferation values higher than CMC90% and at the same time comparable with those of native SOD. This may be due to the fact that CMC50%, thanks to a lower cross-linking degree, has a three-dimensional structure more suitable for the cell life and proliferation. In addition, the highest increase as well as the highest absolute values of fibroblasts proliferation were found for SOD–CMC50%, whereas SOD–CMC90% showed behavior similar to native SOD. These data confirmed as stated before, in fact, in the case of SOD–CMC50% the positive effect on fibroblasts proliferation might be ascribable both to the CMC50% structure and the presence of SOD.

Wound repair test. The increase in primary human skin fibroblast growth and proliferation *in vitro* determined by SOD and SOD–CMC hydrogels encouraged us to test *in vivo* the effect of the native SOD and SOD–CMC hydrogels on skin wound repair of Wistar rats.

Figure 3 demonstrates the cumulative percentage of closed wounds per day during 21 days of macroscopic observation of the experimental and control animals.

The closing of the wounds began on the 12th day in the groups of rats treated with SOD and the SOD–CMC hydrogels. In contrast, it began on the 16th day in the control group.

In the rats treated with SOD and SOD–CMC hydrogels, not only the wound regeneration started earlier but also the percentage of healed animals was greater.

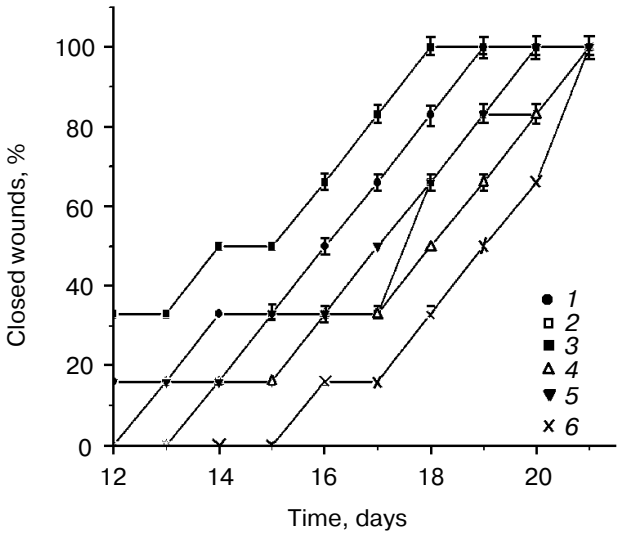

Fig. 3. Cumulative percentage of closed wounds per day during 21 days of macroscopic observation of the experimental and control animals: SOD (*1*), CMC50% (*2*), SOD–CMC50% (*3*), CMC90% (*4*), SOD–CMC90% (*5*), control (*6*). Data from representative experiments performed in triplicate (see "Materials and Methods" section).

Wound repair test results

	Wound healing beginning	% of healed rats at 16th day	Complete wound healing
SOD	12th day	50	19th day
Control	16th day	16	21st day
CMC50%	13th day	33	...
CMC90%	14th day	33	21st day
SOD–CMC50%	12th day	66	18th day
SOD–CMC90%	12th day	33	20th day

In fact, as seen in the table, on the 16th day there was 50% healed rats treated with SOD, 66% of those treated with SOD–CMC50%, the 33% of those treated with SOD–CMC90%, and only 16% of the control.

Furthermore, it was found (table) that the complete wound healing occurred first in the rats treated with SOD–CMC50% (18th day) and then in those treated with native SOD (19th day), SOD–CMC90% (20th day), and the control (21st day). In the groups treated with the gels without enzyme the wound regeneration started earlier than in the control group (13th day for CMC50% and 14th day for CMC90%), but it ended on the same day (21st day).

There was a complete correspondence between the results obtained for the human fibroblasts proliferation *in vitro* test and the wound repair *in vivo* test. In fact, in both cases it was found that SOD conjugated with CMC hydrogels produced the same or even better effects than native SOD.

Physical sorption of SOD into CMC hydrogels to prepare SOD–CMC hydrogel systems can be considered a good strategy to carry the enzyme to injured organs or tissues, where usually excessive reactive oxygen species generation occur and consequently it is necessary to stimulate the cellular proliferation as a way to help tissue regeneration. The obtained data show that the conjugation of SOD with CMC hydrogels increases the SOD-resistance to H_2O_2 inactivation and at the same time has a beneficial effect both on *in vitro* human fibroblast proliferation and *in vivo* rat back skin wound repair process. In particular, these SOD–CMC hydrogels might be suitable for skin reconstruction application.

In fact, it is well known that the reconstruction of skin requires both the dermal and epidermal component of skin. One of the most used approaches consists of the immediate coverage of the burned or wounded skin with fibroblasts and keratinocytes co-culture on a three-dimensional hydrogel support [24-26]. The pore size of the hydrogel permits fibroblast ingrowth and spreading [27], as well as for revascularization after transplantation [28].

Fibroblasts seeded into the porous structure of the hydrogel provide a dermal equivalent suitable to support epidermal cells. On the other hand, it has been showed that local application to thermally injured tissue of rabbit's back skin with enzymatic radical scavengers such as human recombinant SOD has a beneficial effect on the extent of the post burn and producing a significantly quicker re-epithelization to three weeks [29].

In our case hydrogel, in particular the SOD–CMC50%, acts as a support that favors the proliferation of primary human fibroblasts and, stabilizing the SOD, helps open wound healing.

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