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Anti-inflammatory properties of superoxide dismutase modified with carboxymetil-cellulose polymer and hydrogel

A. Chiumiento · A. Dominguez · S. Lamponi · R. Villalonga · R. Barbucci

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Abstract Superoxide dismutase (SOD) was chemically bound to carboxymethyl-cellulose (CMC) polymer.

Furthermore, SOD was also trapped into two hydrogels of CMC with 50% and 90% crosslinking degree. The ability of the two SOD-CMC hydrogels to capture SOD and their release kinetics were investigated. ATR FT-IR spectrometry was used to study the conformation of SOD interacting with both CMC polymer and hydrogels. The effect of SOD-CMC polymer conjugate and SOD-CMC hydrogel systems upon human fibroblasts was studied in vitro measuring the cell proliferation inhibition index and evaluating cell morphology. Using the xanthine oxidase-nitroblue tetrazolium assay, the specific activity of bound SOD to CMC polymer or trapped into hydrogels was evaluated.

The specific activity of the enzyme was higher in SOD-CMC hydrogels than in SOD-CMC polymer conjugates.

1. Introduction

Pharmaceutically active peptides and proteins, such as insulin, growth hormones and cytokines have a large-scale production thanks to the recent advances in biotechnology [1]. Effective administration of protein drugs requires specific delivery methods for several reasons: firstly proteins have a brief span within the blood circulation because they are rapidly degraded by the proteolytic enzymes when they are administrated orally [2]; hence their pharmacological effect can only be obtained by repeated injections. Furthermore.

It's known that oligosaccharide chains provide structural and functional stability to the naturally occurring glicoenzymes [3]. It has been found that the chemical conjugation of enzymes with water-soluble polymers lead to a strong improvement of their stability [4–5]. In particular, Villalonga *et al*. demonstrated that the covalent attachment of ionic polysaccharides, such as carboxymethylcellulose (CMC), to hydrolytic enzymes, as trypsin [6] and invertase [7] enhances their $t_{1/2}$ in circulation and their biological activity. Furthermore, the $t_{1/2}$ extending and the increase of their anti-inflammatory effect have been found for antioxidant enzymes by Veronese *et al*. [8].

With this in mind, we focused our attention on Superoxide-dismutase (SOD). It plays a key role in the cellular response to oxidation stress, catalyzing the dismutation of superoxide free radicals (O_2^-) to hydrogen peroxide and water. The commonly accepted mechanism of reaction involves the cyclic reduction and re-oxidation of Copper by successive molecules of superoxide (Scheme 1) [9].

Furthermore, SOD is very important in many pathological conditions avoiding an over-production of reactive oxygen species which can act as second messenger, activating the synthesis of other mediators involved in the inflammatory process.

In this work we investigated the effect on Bovine SOD stability not only of the chemical conjugation of bovine SOD [10] to the water soluble CMC polymer (Fig. 1), but also of its physical adsorption on CMC hydrogels with different cross-linking degree.

A. Chiumiento · S. Lamponi · R. Barbucci (✉)
CRISMA and Department of Chemical and Biosystem Sciences and Tecnologies, University of Siena, Via A. Moro n. 2, Siena 53100, Italy
Tel.: +39 0577 234382
Fax: +39 0577 234383
e-mail: barbucci@unisi.it

A. Dominguez · R. Villalonga
Grupo de tecnologia enzimatica, Universidad de Matanzas, Matanzas 44740, Cuba

$$O_2 + SOD^{-}Cu^{2+} \longrightarrow SOD - Cu^{+} + O_2$$
$$SOD - Cu^{+} + O_2 \longrightarrow SOD - Cu^{2+} + H_2O_2$$

Scheme 1 Scheme of enzymatic activity of SOD.

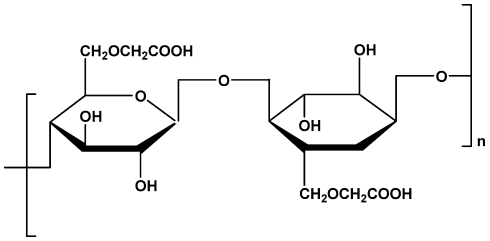


Fig. 1 Carboxymethyl-cellulose disaccharide repetitive unit.

CMC was linked to SOD by covalent conjugation using two different chemical routes: in one case, SOD was linked to CMC by using $NaBH_4$ [6]. In the other case, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide was used as coupling agent [7].

Moreover, SOD was trapped within the CMC hydrogels. Firstly hydrogels of CMC with 50% and 90% cross-linking degree were prepared [11]. Secondly, they were kept in contact with a SOD solution allowing the enzyme uptake on the gel. Then, physical-chemical and biological characterization were performed on both CMC conjugates and hydrogels. The main features of SOD linked to the CMC polymers, such as the secondary structure and the biological activity, were compared with the ones of SOD trapped within the CMC hydrogels.

2. Materials and methods

2.1. Materials

Sodium salt of carboxymethyl cellulose (CMC-Na, viscosity 402 mPa*s w/v aqueous solution at 25 °C, carboxymethylation degree of 0.9±0.1 per monosaccharide unit, $M_w = 100$ KDa), was supplied by Hercules Italia S.p.A. (Italy).

N,N′-dimethylformamide (DMF), 2-Chloro-1-methylpyridinium iodide (CMPJ), formaldehyde solution, tetrabutylammonium hydroxide (TBA), 1,3-diaminopropane, xanthine sodium salt and Xanthine Oxidase, resin Dowex 50WX8 were purchased from FLUKA Chemie AG (Switzerland).

Triethylamine and 1-ethyl-3(3-dimethylaminopropyl) carbodiimide hydrocloride (EDAC) were purchased from MERCK.

Bovine erythrocytes superoxide dismutase (SOD), sodium borohydride ($NaBH_4$), sodium m-periodate ($NaIO_4$), Tripsina-EDTA (1x), Dulbecco's Modified Eagle's Medium (DMEM), Fetal Calf Serum (FCS) and L-Glutamine-Penicillin-Streptomycin solution (200 mM L-Glutamine, 10000 U penincillin and 10 mg streptomycin in 0.9% NaCl) were purchased from SIGMA. DMEM was supplemented with 10% FCS and 1% Glutamine-Penicillin-Streptomycin solution: this medium is further referred to as DMEM complete medium.

2.2. Methods

2.2.1. Synthesis of the SOD-CMC conjugates

SOD-$CMC_{OXID.}$: CMC was previously activated by periodate treatment, as described previously [6]. 25 mg of the SOD were dissolved in 10 mL of 20 mM sodium phosphate buffer (pH 7.2) and then were mixed with 400 mg of activated CMC and stirred at 4 °C in the dark for 1 h. 20 mg of $NaBH_4$ were further added and the reaction mixture, which was kept under continuous stirring for 6 h. The solution was further dialyzed against 20 mM sodium phosphate buffer (pH 7.2).

SOD-CMC_{EDAC}: 160 mg of CMC and 10 mg of SOD were dissolved in 4 mL of 50 mM potassium phosphate buffer (pH 6.0). Then, 20 mg of EDAC were added to this reaction mixture containing the solution was stirred for 1 hour at room temperature and for 16 hrs at 4 °C, subsequently it was dialysed and purified as described previously for the SOD-CMC_{OXID}. conjugate [7].

Polymer content in the SOD-CMC conjugates was determined by quantifying the total amount of carbohydrates by the phenol-sulfuric acid method [12], using glucose as a standard. Protein concentration was estimated as described by Lowry *et al*. [13] using bovine serum albumin as a standard.

2.2.2. Synthesis of SOD-CMC hydrogels

The polysaccharide hydrogels were synthesised as described previously [11]. Briefly, the sodium salt of CMC was converted in TBA salt of CMC, soluble in DMF. Then, a stoichiometric amount of CMPJ was added to the solution to activate 50% and 90% of the carboxylate groups respectively. The reticulation of the hydrogel was obtained using the 1,3 diaminopropane, as cross-linker, together with a small amount of triethylamine as a catalyst, stirring for 3–4 hours and with nitrogen flow at 4 °C. The hydrogel was then dried by lyophilisation. Potentiometric titration was performed on the swollen hydrogel, to check the degree of crosslinking [14].

Later on, 10 mg of hydrogels were immersed in 1 mL of 6,6 mg_{SOD}/ml_{H2O} a solution of SOD for 72 hrs at 4 °C. Excess SOD was removed washing in distilled H_2O at 4 °C. The SOD-hydrogels were then frozen in liquid nitrogen, to prevent conformational changes and lyophilized.

2.2.3. FTIR-ATR analysis

Infrared analysis was performed using the FTIR-ATR spectrometer Bio-Rad FTS 6000 purged with nitrogen. The ATR

spectra of dry samples were recorded with a horizontal (PIKE) ATR accessory equipped with a 45 ° Ge ATR crystal and a mercurium cadmium telluride (MCT) detector. Sixty-four scans at a resolution of 4 cm^{-1} were averaged for each spectrum. Using WIN-IR PRO version 2.6, recorded spectra were elaborated by baseline correction and smoothing (box-car function; 9 N. of P.). Then conformational changes of SOD, due to the interactions with the polysaccharide (polymer and Hydrogel), were studied taking into account the AMIDE I region (1700–1580 cm^{-1}) of the spectrum. The CMC polymer or CMC hydrogel spectrum was then subtracted from the spectrum of the corresponding SOD containing system to obtain the difference in the spectrum of SOD, which showed the secondary structure of the enzyme interacting with the polymer or the hydrogel. These difference spectra were always compared to those of the native enzyme.

The spectra of the SOD-CMC polymer conjugates and of the native dry SOD were Fourier deconvoluted using half-width at half height (HWHH) = 14 and a resolution enhancement factor (K) 2.4. These values were adopted to avoid side-lobes [15].

2.2.4. Scanning Electron Microscopy (S.E.M.) analysis

A Philips XL 20 Scanning Electron Microscope was used for the analysis of hydrogels structure and of adherent cells on cover glasses. Water swollen hydrogels were placed in cryotubes and cooled by liquid nitrogen; after cooling, gels were lyophilised, mounted on SEM stubs and gold-sputtered with an automatic sputter coater (BALTEC SC 050, Balzer).

To evaluate cells morphology, the cover glasses with the formaldehyde fixed cells were dehydrated and left at room temperature for 24 hours with ethanol gradient treatment. They were then mounted on SEM stubs and gold-sputtered with an automatic sputter coater (BALTEC SC 050, Balzer).

2.2.5. Drug release investigation

All the SOD-CMC hydrogels were enclosed in a small bag made of hydrophobic water-permeable net (FALCON cells strainer 100 μm nylon) and immersed in a container with 10 mL water at 4 °C, under stirring. At intervals of 6, 12, 24, 48, 72 and 96 hours, 2 mL of release medium were withdrawn for UV/Visible spectrophotometer analysis and immediately returned to the stock after each measurement. The samples were prepared in triplicate and the measurements were performed with a ULTROSPEC 3300 SPECTROPHOTOMETER PRO at a wavelength of 258 nm.

The amount of SOD released was determined as follows:

$$A = E_{SOD} \times C_{SOD} \times l$$

where: A = absorbance value, E = 10400 (extinction coefficient), l = 1 cm, C = concentration expressed in mg/mL [16].

For *kinetics* analysis of release the following formula was used: $\frac{M_t}{M_0} \Big/ t$

where: M_t = mass of SOD at "t" time and M_0 = mass of SOD at "0" time.

2.2.6. Atomic absorption spectroscopy measurements

The analysis was performed by means the spectrometer Analist 100 HGA-800 PERKIN ELMER equipped with the AUTOSAMPLER AS-72 automatic sampler system. An exact amount of dried sample was totally decomposed in an accurate volume of a mixture 1:1 of HNO_3/H_2O_2. Three Cu^{2+} standard solutions of different and known concentrations were used to obtain the corresponding standard curve. The measurements were performed in triplicate and repeated three times.

2.3. Biological tests

2.3.1. Cell proliferation inhibition index (C.P.I.I.)

To evaluate the *in vitro* cytotoxicity of SOD-CMC polymers and hydrogels, the direct contact test, proposed by ISO 10993-5 Biological evaluation of medical devices – Part 5: Tests for cytotoxicity: *in vitro* methods, was utilised. This test is suitable for materials which have various shapes, sizes or physical states (i.e. liquid or solid) and it has been choosen because the samples to be tested were both liquid (SOD-CMC_{OXID} and SOD-CMC_{EDAC} solutions) and solid (SOD-CMC hydrogels).

Human diploid fibroblasts from skin biopsies of normal individuals were routinely cultured in Dulbecco Modified Eagle's Medium (DMEM, Sigma,USA), containing 10% γ-ray sterilised Fetal Calf Serum (Sigma, USA), 1% L-Glutamine-Penicillin-Streptomycin (Sigma, USA). Cell were harvested by Trypsin-EDTA (1x) (Sigma, USA), centrifuged at 1000 r.p.m. for 10 min. at room temperature and suspended in fresh DMEM. The number of cells was adjusted at 5×10^3/ml. One ml of cell suspension was then seeded into each well of a 24-multiwell plate and incubated for 72 hours at 37 °C in 5% CO_2 until the cells have grown to approximate confluence. Then 1 mL of 3% (w/v) of CMC polymer, SOD-CMC_{EDAC} and SOD-CMC_{OXID} in DMEM (sterilised by 0.2 μm filtration) were added at the cell adhered to the bottom of the 24-multiwell plate. In the same time, 10 mg of each SOD-CMC hydrogel (CMC 50%, SOD-CMC 50%, CMC 90%, SOD-CMC 90%) previously sterilised in EtOH 70% and swollen in DMEM, were carefully placed upon the cell layer. Organo-tin poly (vinyl chloride) was used

Table 1 Structural properties of native and SOD conjugated with CMC polymer.

Sample	CMC content (%, w/w)	Amount of free NH_2 groups (mol/mol)	Mol di SOD per Mol di CMC
Native SOD	–	22	
SOD-CMC_{OXID}	62	11	1.2
SOD-CMC_{EDAC}	53	9	1.8

as positive control material (Portex Ltd. Hythe, Kent, CT21 6JL, UK, Product No 499-300-000). High density polyethylene (U.S. Pharmacopeia (Rockville – Maryland – USA) was used as negative control. All samples were set up in triplicate.

Cell proliferation inhibition index (C.P.I.I.) was evaluated, after an incubation of 72 hrs as previously described by De Groot *et al*. [16]. In particular, media and hydrogels were removed from each well. The cell layers were washed with PBS to remove remaining materials and dead cells. The adhering cells were fixed in 2.5% (v/v) glutaraldehyde in 100 mM sodium cacodylate for 30 min, washed in 100 mM buffer cacodylate for 30 s, rinsed with distilled water. Cell count was performed by inspection with an Olympus BX 40 light microscope. The number of cells was measured before and after considering five different areas for each sample. So C.P.I.I. was calculated as follows:

$$\text{C.P.I.I.} = \left[100\% - \left(\frac{\text{mean cell number of test culture}}{\text{mean cell number of control culture}}\right) \times 100\right] \pm \text{SD}$$

Moreover, cell morphology was evaluated by S.E.M. analysis. For this reason the samples, after cell count, were left standing in dehydration solutions (70% v/v, 90% and absolute ethanol) for total cell dehydration. Finally, the samples were desiccated overnight under vacuum, the bottom of each well was cut by a hot blade and then gold-sputtered. SEM at 15 kV acceleration voltage was used to observe the morphology and to evaluate the perimeter fifty adhered cells for each sample. The collected data were analysed by an NHI Image Program (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov./nhi-image/) and the statistical analysis of the data was performed by OriginPro 7.0 (OriginLab Corporation).

2.3.2. Enzyme assay

SOD activity in the conjugates (SOD-CMC polymer) and in the systems (SOD-CMC hydrogel) was determined by the xanthine oxidase-nitroblue tetrazolium method [18].

Briefly, the reaction mixture consisted of phosphate buffer (0.5 M, pH 7.4), SOD-CMC polymer or SOD-CMC hydrogel (10% w/v); xanthine (1 mM), nitrobluetetrazolium (57 mM) was incubated for 15 minutes at room temperature. Then, the reaction was initiated by the addition of xanthine oxidase (50 mU). The rate of reaction was measured by recording change in the absorbance at 550 nm due to formation of formazan, a reduction product of nitrobluetetrazolium.

3. Results and discussion

3.1. SOD-CMC polymer conjugates

As determined by phenol-sulfuric acid method, the conjugates SOD-CMC_{OXID} and SOD-CMC_{EDAC} contained a different amount of enzyme (Table 1), about 1.2 mol of CMC per 1.8 mol of SOD (these values were obtained considering the repeating unit of both the polysaccharide and the enzyme).

Furthermore, ATR FT-IR spectra demonstrated that SOD in the SOD-CMC_{EDAC} and in the SOD-$CMC_{OXID.}$ conjugates went through the same conformational changes assuming a conformation different from that of native dry SOD (Figs 2a). In fact, native SOD showed a band centred at 1637 cm^{-1} with a shoulder at 1666 cm^{-1}. The band at 1637 cm^{-1} could be assigned to the high frequency twisted β-sheets with weaker hydrogen bonding [19–20]. The band at 1666 cm^{-1} was due to turns, which formed major structural elements of the loops [21–22]. The coupled SOD, in both the two conjugates, was characterized by a predominant low-frequency distorted β-sheet structure with strong intra- and inter-molecular hydrogen boding (1605 cm^{-1}) and by weak components at 1650 and 1630 cm^{-1} associated to the α-helix and regular main core β-sheet domains respectively [15, 19, 20]. As it can be seen in the Fourier deconvoluted spectra (Fig. 2b) the α-helix component is also present in the native dry SOD (in accordance with the previous findings by Lo *et al*.[15]). Consequently, the conformational changes from the native to the conjugate SOD were only related to the shift of the β-sheets components to lower wavenumbers.

3.2. SOD-CMC hydrogel

CMC-based hydrogels were synthesised with 50% and 90% cross-linking degree (i.e. percentage of the number of COO^-, present in the polysaccharide chain, involved in the cross-linking reaction). Scanning electron microscopy analysis showed that CMC 50% was characterized by randomly distributed holes of variable diameter (Fig. 3a), while CMC 90% had a more compact structure, formed by layers (Figs. 3b).

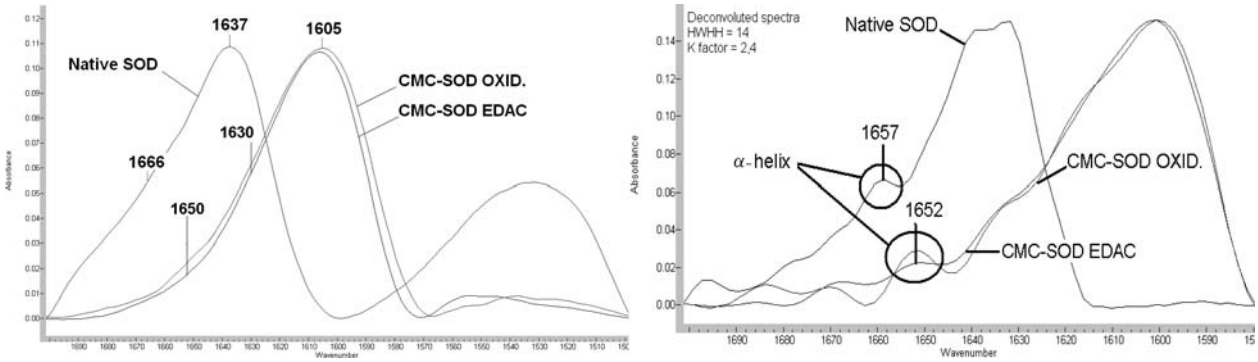


Fig. 2 SOD spectra of native dry SOD, SOD-$CMC_{OXID.}$ and of SOD-CMC_{EDAC} conjugates(2a); deconvoluted (HWHH = 14, K_{factor} = 2.4) spectra of native SOD, SOD-$CMC_{OXID.}$ and of SOD-CMC_{EDAC} conjugates (2b).

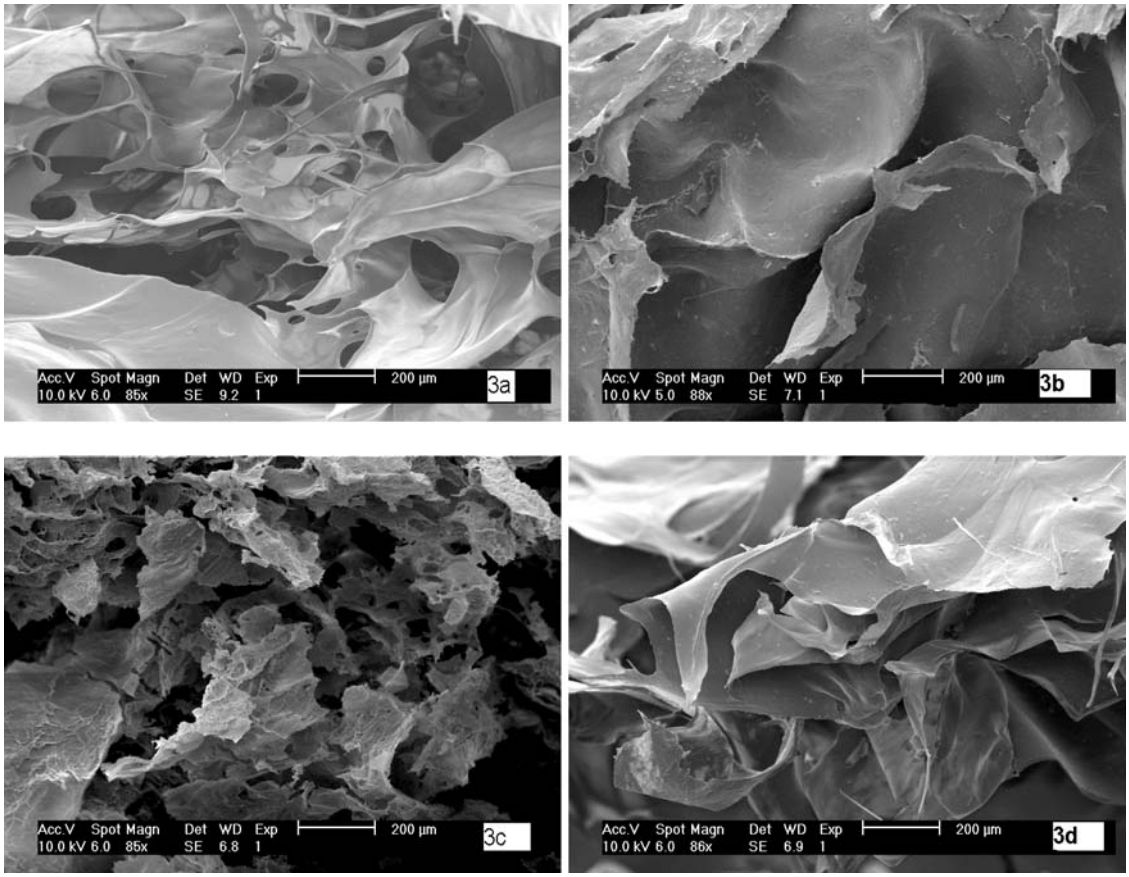


Fig. 3 Scanning electron microscope image of CMC 50% (3a) and CMC 90% (3b); scanning electron microscope image of SOD-CMC 50% (3c) and SOD-CMC 90% (3d).

The presence of SOD did not determine noticeable changes in the structure of the SOD-CMC 90% (3d), while the morphology of SOD-CMC 50% became spongy with small diameter holes (3c).

The two CMC hydrogels retained a different amount of SOD: CMC 50% captured an amount of SOD ≈5 times greater than CMC 90% (table 2).

These amounts of SOD were released by the two hydrogels with the same kinetics (Fig. 4), even though the SOD-CMC 50% reached *the plateau* (the point at which the system SOD-hydrogel releases no further SOD) in 48 hours, about 24 hours in advance with respect to SOD-CMC 90%.

The ATR FT-IR analysis of SOD tightly interacting with the hydrogel was performed at the plateau, i.e. after 48 hours for the SOD CMC 50% and after 72 hours for the SOD-CMC 90%. The amide I spectrum of SOD interacting with CMC 50% showed two bands (Fig. 5a): the main band at

Table 2 Amount of SOD retained at *the plateau* by CMC 50% and 90% hydrogel.

Sample	Conc. of Cu^{2+}	Conc. of SOD	Conc. of SOD after release
SOD-CMC 50%	$2.16 \times 10^{-3} \pm 0.02$	$6.55 \times 10^{-1} \pm 0.03$	$2.73 \times 10^{-1} \pm 0.02$
SOD-CMC 90%	$0.46 \times 10^{-3} \pm 0.08$	$1.40 \times 10^{-1} \pm 0.06$	$0.88 \times 10^{-1} \pm 0.06$

1640 cm^{-1} referred to SOD β-sheets domains with weaker hydrogen bonding, present in the native SOD too; and the weaker one at 1600 cm^{-1} due to SOD low-frequency anti-parallel β-sheets domains with stronger hydrogen bonding (intra-molecular and intermolecular at the interface with CMC) [18–19]. The CMC 90% had instead promoted noticeable conformational changes of the enzyme chains. The spectrum of SOD interacting with CMC 90% hydrogel showed a band at 1666 cm^{-1}, associated with the turns of the loops; a band at 1640 cm^{-1}, due to the β-sheets domains not-interacting with CMC, and the strongest band, at 1604 cm^{-1}, which gave the greatest contribution to the enzyme conformation, related to SOD anti-parallel β-sheets domains with strong hydrogen bonding [19–20]. Since the IR spectrum of SOD interacting with the CMC 50% hydrogel is similar to that of the native enzyme, we may reasonably suppose that the native SOD conformation is almost retained by the interacting enzyme. This finding could also explain the fact that the SOD was released more easily by the CMC 50% than by the CMC 90% hydrogel.

3.3. Biological tests

To investigate the effect of the samples on the proliferation of human fibroblasts, the proliferation inhibition index (C.P.I.I.) and the cell morphology, evaluated in terms of both surface and perimeter, were determined. These results were summarized in tables 3, 5 and Figs. 6 and 7. Furthermore, the enzymatic activity of native free SOD and SOD in CMC-based systems was evaluated and compared *in vitro* by the xantine oxidase-nitroblue tetrazolium assay (Tables 4 and 6).

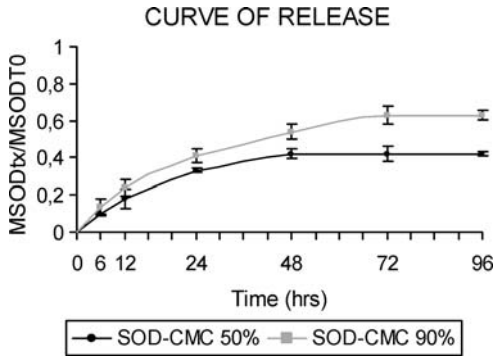


Fig. 4 Kinetics of release of SOD-CMC hydrogels.

Table 3 Cells proliferation inhibition index values of SOD-CMC conjugates.

Sample	CPII %±SD	Surface (μm^2)	Perimeter (μm)
PVC	100 ± 0	0	0
Cover glass	2 ± 1	3888±35	533±14
Native SOD	14 ± 1	3539 ± 40	491 ± 33
CMC Polymer	47 ± 6	1345 ± 14	548 ± 43
$SOD\text{-}CMC_{OXID.}$	8 ± 2	2598 ± 17	397 ± 16
$SOD\text{-}CMC_{EDAC}$	15 ± 2	2565 ± 20	360 ± 21

3.4. SOD-CMC polymer conjugates

As reported in Table 3, in the presence of a CMC polymer solution the C.P.I.I. value was 47 and the fibroblast cells occupied a low surface area (1345 μm^2) with the highest value of perimeter (548 (m).

Therefore, as cell morphology is tightly correlated to the biocompatibility characteristic of the substrate and/or of the soluble molecules in contact with cells, we may hypotise that the CMC polymer in the culture medium worsened the cells life conditions, so that they reduced their surface contact with the cover glasses and developed few but long philopodia (Fig. 6a). This behaviour is distinctive of a state of cells devoted to migrate onto a "better surface". On the contrary, the C.P.I.I. value of a SOD solution was low and cells changed their shape, obtaining a larger contact area (Fig. 6b). Anyway, in both the samples, cytoplasm is pitted and cells have some debris.

Both the two conjugates induced an inhibition of fibroblasts proliferation lower than the native CMC polymer and comparable to that of native SOD (Table 3). Fibroblasts in contact with $CMC\text{-}SOD_{EDAC}$ and $CMC\text{-}SOD_{OXID}$. showed a good adhesion area. Thus, we could state that the SOD coupling to CMC polymer improved fibroblast cell life condition in comparison with the CMC polymer.

Concerning the *in vitro* specific activity, it was observed that SOD-CMC conjugates had a less enzymatic activity than that of native SOD: $SOD\text{-}CMC_{EDAC}$ retained the 78% of the native SOD activity, while the $SOD\text{-}CMC_{OXID.}$ retained only the 68% (table 4). This behaviour might be attributed to the different amount of the enzyme coupled to the CMC polymer by using the two mentioned chemical routes.

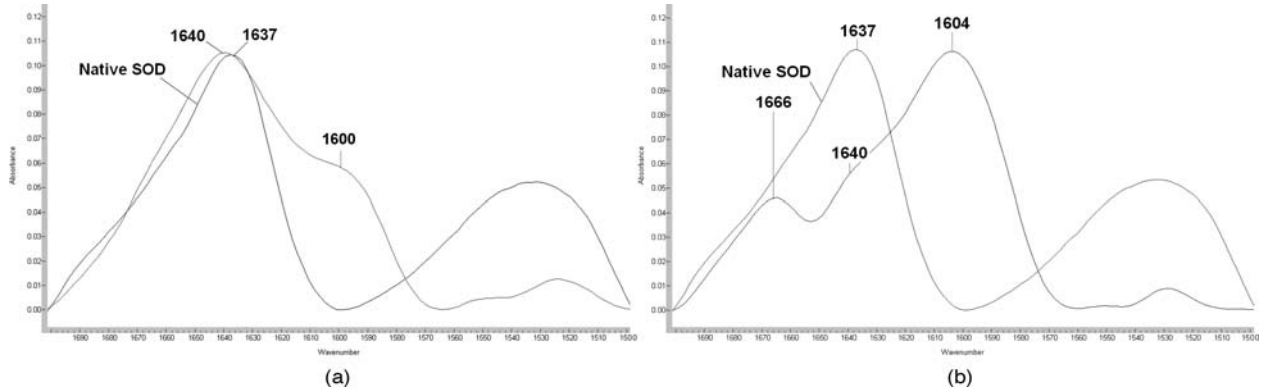
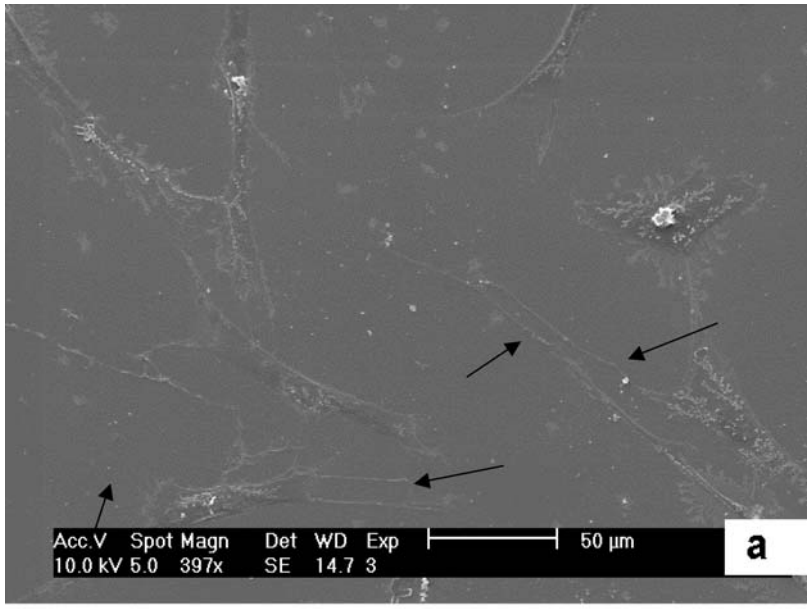


Fig. 5 Conformation of native dry SOD; of SOD in SOD-CMC 50% hydrogel (5a); of SOD in SOD-CMC 90% hydrogel (5b).



Arrows indicate long pseudopodia produced by cells.

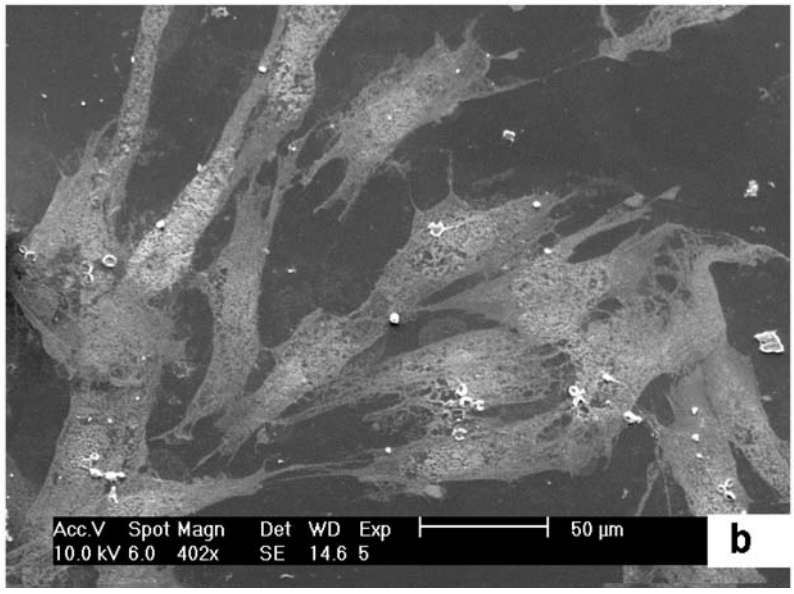


Fig. 6 (a) S.E.M. image of cells morphology on CMC polymer**:** the cells minimize their surface contact with the cover glasses and develop few but long pseudopodia; (b) S.E.M. image of cells morphology on SOD: the cells show a large contact area and many pseudopodia.

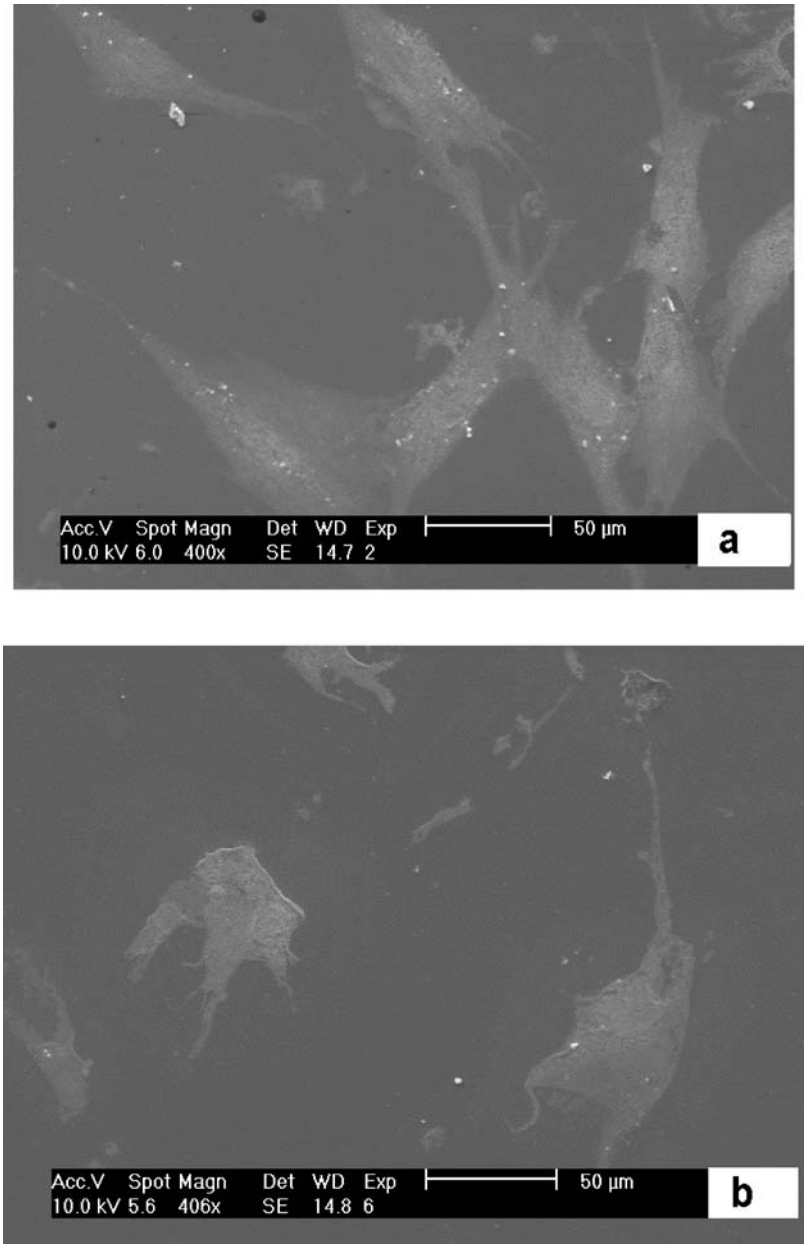


Fig. 7 (a) S.E.M. image of cell morphology on SOD-CMC 50% hydrogel: The cells present of a very big adhesion area on the cover glasses; (b): S.E.M. image of cell morphology of SOD-CMC 90% hydrogel: the cells show a lower contact area with cover glasses and fewer pseudo-podia than SOD-CMC 50%.

Table 4 Evaluation of the enzymatic activity of SOD in SOD-CMC conjugates.

Sample	Specific activity (U/mg)
Native SOD	3500 ± 12
SOD-CMC_{OXID}. conjugate	2400 ± 09
SOD-CMC_{EDAC} conjugate	2735 ± 17

Table 5 Cells proliferation inhibition index values of SOD-CMC hydrogel systems.

Sample	CPII[1] %±SD	Surface (μm^2)	Per.[1] (μm)
Native SOD	14 ± 1	3539 ± 40	491 ± 33
CMC 50%	16 ± 3	1877 ± 12	382 ± 18
SOD-CMC 50%	4± 2	4131 ± 18	516 ± 30
CMC 90%	31 ± 2	2432 ± 35	364 ± 18
SOD-CMC 90%	20 ± 7	2539 ± 27	339 ± 13

3.5. SOD-CMC hydrogel

CMC Hydrogels with 50% and 90% cross-linking degree showed C.P.I.I.s lower than their corresponding polymer. These values were further reduced after SOD addition (table 5).

The C.P.I.I. of SOD-CMC 50% was the lowest of the series, very similar to that of cover glass (negative control). It completely agreed with the SOD-CMC 50% highest average value both of surface and perimeter of cells (Fig. 7 a):

Table 6 Evaluation of the enzymatic activity of SOD in SOD-CMC hydrogel systems.

Sample	Specific activity (U/mg)
Native SOD	3500 ± 12
SOD-CMC 50% Hydrogel	3000 ± 11
SOD-CMC 90% Hydrogel	2800 ± 15

the cells on SOD-CMC 50% presented a very large adhesion area on the cover glasses. Instead, the cells on the SOD-CMC 90% showed a smaller contact area and fewer pseudo-podia (Fig. 7b).

As showed in Table 6, both hydrogels had less in vitro anti-inflammatory activity than the free dry SOD. SOD-CMC 50% also showed a better specific activity, 86% of free SOD anti-inflammatory activity, than the SOD-CMC 90%, which retained the 80% of SOD activity.

4. Conclusions

In this paper the biological activity of SOD coupled with two CMC based systems was studied and compared with that obtained by trapping the enzyme into two CMC hydrogels.

The covalent bond of SOD to the CMC polymer determined strong and similar three-dimensional rearrangements of the enzyme in both the conjugates. Consequently, the different biological response between the two conjugates cannot be related to the conformational changes occurred in the enzyme after the binding to CMC polymer; but it may be connected to the different amount of SOD coupled to CMC using the two chemical routes. Although the SOD in both the two conjugates showed a lower activity than free native SOD, the SOD-$CMC_{OXID.}$ and SOD-CMC_{EDAC} conjugates were more resistant to inactivation by H_2O_2, in comparison with native SOD. Thus, the decrease of the enzymatic activity was completely balanced to the improvement of functional stability of the enzyme.

However, the *in vitro* anti-inflammatory activity was better preserved when the enzyme was trapped in CMC hydrogels rather than when it is chemically linked to the polymeric compounds.

The CMC 90% hydrogels interacted with the enzyme promoting noticeable conformational changes with a consequent loss of enzymatic specific activity, while the CMC 50% hydrogel interacted with the enzyme determining smaller conformational changes and retaining the great part of the of free SOD specific activity (86%). Furthermore, the different morphology of the two hydrogels deeply influenced the ability of up-taking and retaining SOD: CMC 50% trapped an amount of the enzyme 5 times greater than the CMC 90% and at *the plateau* retained an amount of SOD 3 times greater than the CMC 90%. As a consequence, SOD-CMC 50% hydrogel at the plateau showed the highest *in vitro* specific activity among the tested samples. In conclusion, we can state that the physio-sorption of bovine SOD on CMC hydrogels can be considered a strategy for enzyme stability enhancement more suitable and effective than the usually used chemical conjugation. In particular CMC 50%, had a better *in-vitro* anti-inflammatory activity and C.P.I.I. values than the CMC 90% hydrogel and the CMC polymers covalently conjugated.

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