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# **Exploring the Effect of Pore Size on the Activity of Superoxide Dismutase Mimics Immobilized in Mesoporous Spherical Silica Particles**<sup>†</sup>

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The imidazolate-bridged diCu(II) complex  $[Cu_2(dien)_2(\mu-Im)]^{3*}$  (Im = imidazolate, dien = diethylenetriamine) was encapsulated in mesoporous spherical silica particles (MSSP) with retention of the morphology and mesostructure of the guest-free silica particles. This hybrid material catalyzes the dismutation of superoxide efficiently, with second-order catalytic rate constant of 8.28 x 10<sup>7</sup> M<sup>-1</sup> s<sup>-1</sup>, obtained from the nitro blue tetrazolium photoreduction inhibition superoxide dismutase assay, at pH = 7.8. The small silica channels of MSSP ( $w_p = 2.7$  nm) enhance the distortion of the Cu(II) local environment compared to mesoporous silicas with wider pores and ensure the two metal moieties of the reduced catalyst remain in close proximity to react with superoxide. These two combined effects increase the SOD activity of the hybrid material ten times over the free catalyst. The small channel size is also relevant for the salpn derived functional SOD mimic [CuZn(salpn)Cl<sub>2</sub>] (H<sub>2</sub>salpn = 1,3-bis(salicylideneamino)propane), which upon encapsulation in MSSP doubles its SOD activity.

#### 1. Introduction

Superoxide dismutase enzymes (SODs) are the main endogenous defense against O2\*. Among them, CuZn-SOD catalyzes the dismutation of  $O_2^{\bullet-}$  with second order rate constant around 10<sup>9</sup> M<sup>-1</sup> s<sup>-1</sup>, being one of the fastest reactions catalyzed by enzymes.<sup>1</sup> The use of SOD as a therapeutic agent to treat diseases caused by reactive oxygen species imbalance is limited by its size and charge (which makes it difficult to enter the cell), and its rapid elimination from the body.<sup>2</sup> For this reason, low molecular weight mimics are important targets as catalytic antioxidants to reduce oxidative stress injuries.<sup>3-5</sup> However, in solution, SOD mimics may undergo hydrolysis, metal dissociation or oligomerization processes during the reaction.<sup>6,7</sup> Encapsulation of the catalyst in a mesoporous solid has proved to be a good strategy for its confinement and site isolation, while preserving the properties of the homogeneous system.8-10 Among the solid supports, mesoporous silica particles (MSP) possess a large contact surface and pore volume which allow high catalyst loading,<sup>11,12</sup> chemical and mechanical stability,<sup>13,14</sup> biocompatibility<sup>15</sup> and controllable geometric

59 60 parameters that enable a suitable design of different types and sizes of pores.<sup>11</sup> In previous works we have reported that encapsulation in MSPs improves the SOD activity of imidazolato-bridged CuZn- and CuCu-mimics,16,17 and that the SOD activity doubled as the pore size of MSP reduced from 8.6 to 3.4 nm. Aimed at verifying if a closer fitting of the silica channels size to the catalyst dimension can be used to further enhance the SOD activity of these complexes, in this work we prepared mesoporous spherical silica particles (MSSP) with pore diameter smaller than the previous ones, and evaluated the SOD activity of  $[Cu_2(dien)_2(\mu-Im)(ClO_4)_2]ClO_4$  (1) (Im = imidazolate, dien = diethylenetriamine), shown in Figure 1, encapsulated in MSSP (1@MSSP). Besides, the catalytic behavior of 1@MSSP was compared to 2@MSSP (2 = [CuZn(salpn)Cl<sub>2</sub>], where H<sub>2</sub>salpn = 1.3 bis(salicylideneamino)propane, in order to ascertain if the small channel size can be also relevant for salpn derived functional SOD mimics.

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### 2. Experimental

#### 2.1. Synthesis of complexes



Figure 1. Complexes used in this work

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#### Page 2 of 7

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Journal of Chemis

#### ARTICLE

[Cu<sub>2</sub>(dien)<sub>2</sub>( $\mu$ -Im)(ClO<sub>4</sub>)<sub>2</sub>]ClO<sub>4</sub> (**1**), and [CuZn(salpn)Cl<sub>2</sub>] (**2**) were synthesized as previously described in reference.<sup>18</sup> Anal. Calc. for Cu<sub>2</sub>C<sub>11</sub>Cl<sub>3</sub>N<sub>8</sub>H<sub>29</sub>O<sub>12</sub>: C 18.9, H 4.2, N 16.0, Cu 18.5%; found: C 18.6, H 4.4, N 15.8, Cu 18.1%. Molar conductivity (DMF) = 200 Ω<sup>-1</sup> cm<sup>2</sup> mol<sup>-1</sup>. Anal. Calc. for CuZnC<sub>17</sub>Cl<sub>2</sub>H<sub>16</sub>N<sub>2</sub>O<sub>2</sub>: C 42.5, H 3.5, N 5.8, Cu 13.2, Zn 13.6%. Found: C 42.5, H 3.3, N 5.7, Cu 13.8, Zn 13.6%. Molar conductivity (DMF) = 5.6 Ω<sup>-1</sup> cm<sup>2</sup> mol<sup>-1</sup>.

#### 2.2. Synthesis of MSSP

The mesoporous silica was synthesized through a modification of the procedure described by Grun et al.<sup>19</sup> employing 98% tetraethyl orthosilicate (TEOS), 99% cetyltrimethylammonium bromide (C16TAB), and NH<sub>4</sub>OH, in a mixture of water and ethanol. TEOS (5 ml, 22.1 mmol) was added to a vigorously stirred mixture of 30 ml of aqueous NH<sub>4</sub>OH (25-30 % wt %;  $\rho$ : 0,88 g/ml) and 2.5 g (6.86 mmol) of C16TAB in 50 ml of distilled water and 75 ml of ethanol. The reaction mixture was stirred at 30 °C for 2 h. The white precipitate was filtered off and washed with copious amounts of distilled water. To remove the C16TAB, the sample was calcined at 550 °C in air for 2 hours, with a heating ramp of 5 °C per min.

#### 2.2. Encapsulation of complexes 1 – 2 in MSSP

0.3 g of the mesoporous silica particles were added to a solution of complex **1** or **2** (0.20 mmol) in 20 mL of methanol. The mixture was stirred for 24 h at room temperature, filtered, and washed with methanol. The solid material was re-suspended in methanol and stirred overnight. The solid was filtered and dried under vacuum. Thermogravimetric analysis: weight loss below 200 °C: 10% (**1**@MSSP) and 3% (**2**@MSSP); weight loss between 200 and 600 °C: 3.8 % (**1**@MSSP); 16.3 % (**2**@MSSP). Metal Anal. (wt %): Cu 1.3 (**1**@MSSP); Cu 2.7, Zn 2.8 (**2**@MSSP). Significant IR bands (KBr,  $\nu$  cm<sup>-1</sup>) for **1**@MSSP: 1640 ( $\delta$ , H-O-H), 1080 ( $\nu_{as}$ , Si–O), 795 ( $\nu_{s}$ , Si–O), 463 ( $\delta$ , Si-O-Si); for **2**@MSSP: 2842/2952 ( $\nu$ , C-H), 1640 ( $\delta$ , H-O-H), 1619 ( $\nu$ , C=N), 1589/1558 ( $\nu$ , Ar), 1080 ( $\nu_{as}$ , Si–O), 795 ( $\nu_{s}$ , Si–O), 463 ( $\delta$ , Si-O-Si).

#### 2.3. Physical measurements

UV-visible spectra were registered with a JASCO V-550 spectrophotometer. The spectra were collected in the range of 300 to 900 nm. EPR spectra of solid samples were obtained on an Elexsys E 500 Bruker spectrometer, operating at a microwave frequency of approximately 9.5 GHz, at 120 K. IR spectra were recorded on a Perkin-Elmer Spectrum One FT-IR spectrophotometer. Solid samples of silica, neat complexes and hybrid materials were run in ATR (attenuated total reflectance) mode on a diamond crystal. Thermogravimetric analyses (TGA) were carried out on a TGA 51 Shimadzu thermogravimetric analyzer. The heating rate was 10°C min<sup>-1</sup>, and the carrier gas (air) flow rate was 50 mL min<sup>-1</sup>. Porosity and surface area were determined from  $N_2$  adsorption-desorption isotherms obtained at 77 K on a Micrometric ASAP 2020 V4.02 (V4.02 G) apparatus. Samples were degassed at 10<sup>-3</sup> Torr and 200 °C for 6 h prior to the adsorption experiment. Surface area (S<sub>BET</sub>) were calculated using the Brunauer-Emmett-Teller (BET)<sup>20</sup> equation over the pressure range  $(p/p^{\circ})$  of 0.05-0.20. The volume of micropores and mesopores ( $V_{\mu P}$  and  $V_{M P})$  was determined by the alpha-plot method<sup>20</sup> using the standard Licrospher isotherm. The total pore

volume (V<sub>TP</sub>) was determined with the Gurvich rule<sup>20</sup>/at 0.98 p/p<sup>2</sup> The pore size distributions were calculated using The Willarder Bezerra–Sapag (VBS) model,<sup>21</sup> on the desorption branch of the N<sub>2</sub> isotherms. The size and morphology of solid materials were recorded on metallized samples using an AMR 1000 Leitz scanning electron microscope (SEM) operated at variable accelerating voltages and with EDX detector NORAN System SIX NSS-200. Transmission electron microscopy (TEM) analysis was performed with a TEM/STEM JEM 2100 Plus with operational voltage of 200 kV (variable), with a LaB6 filament. The zeta potential of the particles was measured with a Horiba SZ-100 nanoparticles analyzer, in triplicate, by using 0.01 wt % dispersions in MilliQ water. HCl and NaOH were used for measurements at different pH values. Conductivity measurements were performed using a Horiba F-54 BW conductivity meter, on 1.0 mM solutions of the complexes in DMF. Metal content was determined using an Inductively Coupled Plasma Mass Spectrometer (ICP-MS) Perkin Elmer NexION 350×.

#### 2.4. Indirect SOD assay

The SOD activity of the free and immobilized complexes was evaluated by measuring the nitro blue tetrazolium (NBT) photoreduction, using a method slightly modified from the originally described by Beauchamps and Fridovich.<sup>22</sup> The suspensions were prepared in 50 mM phosphate buffer of pH 7.8, riboflavin (3.4  $\mu$ M), methionine (10 mM), NBT (46  $\mu$ M) and different amounts of adsorbed complexes (0-5 µM). Riboflavin was last added and the reaction was initiated by illumination of the mixtures with an 18 W fluorescent lamp placed at 15 cm distance, at 25°C.<sup>23</sup> The reduction of NBT was measured at 560 nm after an illumination period of 15 min. The IC<sub>50</sub> values (the concentration of the SOD mimic that induces a 50% inhibition of the reduction of NBT) were determined from concentrationdependent plots. Control reactions confirm that the compounds did not react directly with NBT or riboflavin. Inhibition percentage was calculated according to:

$$IC = \frac{\left[(\Delta Abs/t)_{without \ catalyst} - (\Delta Abs/t)_{with \ catalyst}\right] \times 100}{(\Delta Abs/t)_{without \ catalyst}}$$

#### 3.1. Preparation and characterization of hybrid materials

Mesoporous spherical silica particles (MSSPs) were synthesized employing TEOS as Si source, C16TAB as cationic surfactant and structure directing, and NH<sub>4</sub>OH to generate the basic medium, in a mixture of water and ethanol, the last to favor the formation of spherical particles.<sup>19</sup> The obtained MSSPs were used to host complexes **1** and **2** to render the hybrid materials **1**@MSSP and **2**@MSSP. Compound **1** behaves as **1**:3 electrolyte in solution, as evidenced by the molar conductivity of 200  $\Omega^{-1}$  cm<sup>2</sup> mol<sup>-1</sup> measured in DMF,<sup>24</sup> and is retained inside the channels of MSSPs through strong electrostatic interactions with the negatively charged Si-O<sup>-</sup> groups of the pores surface, while for neutral complex **2**, weaker interactions with the silica surface are expected.

The textural properties of MSSPs and the hybrid materials were analyzed by nitrogen adsorption-desorption measurements at

Page 3 of 7

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#### ARTICLE



Figure 2.  $N_2$  Adsorption-Desorption Isotherms of mesoporous materials at 77 K

77 K. As shown in Figure 2, the three samples exhibit type IV isotherms with a sharp increase at low relative pressure  $p/p^0 =$ 0.3 due to capillary condensation in cylindrical mesopores, typical of strong adsorbent-adsorbate interactions. The absence of hysteresis loop indicates reversible condensation/evaporation in the mesopores.<sup>20</sup> None of the samples contains any meaningful amount of micropores and the plateau after the steep step indicates the lack of significant secondary mesoporosity. Textural data of the mesoporous materials are summarized in Table 1. The MSSP pore size of 2.7 nm is suitable for the incorporation of complexes 1-2 which are about 1.25 and 1.02 nm wide, respectively.18,25 The uptake of the catalyst causes the decrease of BET surface area, total pore volume and average pore diameter, indicating the pore filling with the compound, and leaves the overall shape of the BET curves almost unchanged suggesting the mesoporous structure remains practically unaltered. The marked decrease in the mesopore volume and overall adsorption volume of 2@MSSP is in agreement with the higher proportion of 2 in the hybrid material compared to 1@MSSP (Table 1, last column), determined by ICP and TG analyses.

The mesoscopic ordering of MSSP and 1@MSSP was characterized by low angle X-ray diffraction (XRD). The XRD pattern of MSSP (Figure 3) is typical of ordered mesoporous materials of the type of MCM-41 silica,<sup>26</sup> with a well-defined (100) peak at  $2\theta = 2.36^{\circ}$ , corresponding to an interplanar spacing  $d_{100} = 4.16$  nm, and weak higher order reflections in the  $2\theta$  range of  $4 - 5^{\circ}$  (Figure S1). A wall thickness of 2.10 nm was estimated from  $d_{100}$  and pore diameter determined from sorption isotherms.<sup>27</sup>

Table 1: Textural properties, zeta potential and complex content of hybrid materials



Figure 3. Low angle (bottom) and high angle (top insets) X Ray diffractograms of mesoporous materials

Encapsulation of complex **1** in MSSP shows a 15% decrease of the intensity in the  $d_{100}$  peak and higher order reflections, attributed to a slight decrease of periodicity, while preserving the mesoporosity.<sup>28</sup> The high angle X-ray diffractograms of **1**@MSSP show no peaks associated to crystallites of the complex on the particles surface (Figure 3, left inset), confirming that **1** is essentially located inside the silica pores. Unlike **1**@MSSP, the featureless low angle X-Ray diffractogram of **2**@MSSP suggests that in this case the entrances of the channels are blocked by the complex molecules. Besides, the high angle XRD pattern of **2**@MSSP agrees with that of the free complex (Figure 3, right inset), evidencing that a proportion of **2** is located at or near the surface of the pore opening.

The morphology and size of the mesoporous materials were analyzed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The SEM and TEM images of MSSP, **1**@MSSP and **2**@MSSP are shown in Figure 4. MSSPs possess regular spherical shape and appear unchanged after encapsulation of the complexes, with circularity (defined as the ratio of the largest to smallest particle diameter) of 0.996 (MSSP), 0.993 (**1**@MSSP) and 0.998 (**2**@MSSP). The average particle size of the three materials, calculated from statistical analysis of SEM images of selected particles, was 673 ± 191 nm for MSSP, 807 ± 197 nm for **1**@MSSP, and 643 ± 174 nm for **2**@MSSP. The corresponding histograms are shown in Figure S2.

The zoom-in TEM image of a whole small particle of **2**@MSSP (Figure 4 (f)) shows a structure of open porosity, with a diverging pore channel arrangement toward the particle surface with small ordered areas with honeycomb-like structure observed in the zone where the electron beam is parallel to the

	S <sub>вет</sub> [m <sup>2</sup> g <sup>-1</sup> ]	$V_{\mu P}$ [cm <sup>3</sup> g <sup>-1</sup> ]	V <sub>MP</sub> [cm <sup>3</sup> g <sup>-1</sup> ]	V <sub>TP</sub> [cm <sup>3</sup> g <sup>-1</sup> ]	W <sub>P</sub> [nm]	ζ (mV)	mmol complex/100 g material
MSSP	952	0	0.73	0.78	2.7	-77.6	-
<b>1</b> @MSSP	904	0	0.63	0.69	2.6	-74.6	10.5
<b>2</b> @MSSP	674	0	0.44	0.50	2.1	-53.4	43.4

 $V_{TP} = V_{\mu P} + V_{primary MP} + V_{secondary MP}$ , MP = mesopore;  $\mu P$  = micropore;  $\zeta$  = zeta potential

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## ARTICLE

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channels (Figure S3). Estimated pores and wall widths are  $\approx 2$  nm, in agreement with XRD and adsorption/desorption  $N_2$  isotherms results. The small size of pores and walls thickness weaken these particles. Therefore, the porous structure collapses after prolonged exposure to both X-radiation and electron beams. This behavior differentiates MSSPs from MSPs with larger pores and thicker walls that are more resistant to both X-radiation and electron beams.

MSSP possess a negative zeta potential ( $\zeta$ ) at pH > 2 due to deprotonation of the silanol groups on the surface,<sup>29</sup> with high electrostatic stability ( $|\zeta|$  > 30 mV) in the 4 < pH < 9 range. Zeta potentials of MSSP and hybrid materials measured from Milli-Q water suspensions are shown in Table 1. These materials can be easily resuspended in water showing excellent colloidal stability. Because of the small pore diameter, encapsulated **1** modifies the ionic conditions inside the channels but has little effect on the surface charge properties of MSSP.<sup>30</sup> Unlike **1**, immobilization of complex **2** decreases the absolute value of the

average zeta potential by 24 mV, as the consequence of its higher loading and its presence at the pore openings surface. The thermal degradation pattern (Figure S4) of 1@MSSP and 2@MSSP shows a two-stage mass loss, the first below 150°C corresponding to the loss of water molecules and the second weight loss from 350 to 550°C for 1@MSSP and around 380°C for 2@MSSP, ascribable to the decomposition of the organic ligand. Compared to the free complexes (250°C (1) and 310°C (2)),<sup>18</sup> the decomposition of the encapsulated complexes occurs at a higher temperature. A similar enhancement of the thermal stability of the metal complex on encapsulation had been observed earlier.<sup>16,17</sup> Weight loss data together with the results of metal analyses were employed to calculate the complex loading in the hybrid materials (Table 1, last column). The lower proportion of 1 encapsulated in MSSP agrees with the smaller decrease in the pore volume and surface area (Table 1) determined for this complex.

FT-IR spectra of the hybrid materials show strong bands belonging to the Si-O-Si framework (Figure 5).







(e)



(f)

Figure 4. SEM images of (a) MSSP, (b) 1@MSSP, (c) 2@MSSP; and TEM micrographs of (d) MSSP, (e) 1@MSSP, (f) 2@MSSP

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Figure 5. FT-IR spectra of the mesoporous materials and solid complexes

In the case of **1**@MSSP, IR bands characteristic of functional groups present in the complex are negligible or not observed, probably because the strong bands of the silica matrix hide those from the complex. For **2**@MSSP, where the catalyst is present in higher proportion, the vibrations characteristic of the salpn ligand are not completely masked by the silica matrix bands and can be observed in the 2840-2950 and 1620-1400 cm<sup>-1</sup> spectral regions.

Low-temperature X-band EPR spectra of complexes 1 and 2, free and encapsulated in MSSPs, provided details on the geometry and coordination environment of the Cu(II) ions in the hybrid materials. Complex 1 contains two Cu(II) centers separated by ~ 5.8 Å.<sup>25</sup> The EPR signal of powdered **1** is broadened due to dipolar and spin-spin intermolecular interactions, so that the structural information of the hyperfine coupling between the unpaired electron and the copper ions is lost (Figure 6(left), black line). The encapsulation of the complex in MSSP (Figure 6(left), pink line), results in the dilution of the paramagnetic centers in the diamagnetic matrix giving a welldefined axial EPR signal for 1@MSSP, with spectral parameters  $g_{\perp}$  = 2.05,  $g_{//}$  = 2.25,  $A_{//}$  = 176 x10<sup>-4</sup> cm<sup>-1</sup>, and  $g_{//} / A_{//}$  = f = 128 cm, characteristic of Cu(II) ions in a distorted tetragonal geometry.<sup>31</sup> The lack of  $\Delta Ms = \pm 1$  transitions belonging to the triplet state of a Cu(II)<sub>2</sub> system indicates that the two copper ions are not interacting, which suggests the silica channels constrain the geometry of the  $\mu$ -imidazolate-Cu(II)<sub>2</sub> core enhancing the distortion of the local Cu(II) environment, and modify the relative orientation of the two copper coordination planes.<sup>32</sup> The EPR spectrum of powdered 2 (Figure 6 (right), black) shows a central signal at  $g_1 = 2.08$  flanked by the  $\Delta Ms =$  $\pm 1$  transitions from the triplet state of a spin-coupled Cu(II)<sub>2</sub> system  $(g_2, g_3)$ , which suggests the formation of dimers in the powder.

The spectrum of **2**@MSSP (Figure 6 (right), blue line) shows that immobilization diminishes intermolecular interactions between



Figure 6. Low temperature X-band EPR spectra of solid complexes and hybrid materials

paramagnetic centers affording an axial EPR spectrum typical of an uncoupled Cu(II) center with  $g_{\perp} = 2204^{\circ}dA2^{\circ}e^{3/N}=02642^{\circ}$ . However, the EPR signal of the hybrid is still broad, probably due to the overlap of species with slightly different geometries inside and at the entrance of the silica pores.

#### 3.2. SOD-like activity

The ability of the encapsulated complexes, 1@MSSP and 2@MSSP, to react with O2<sup>--</sup> was evaluated and their activity compared to the reported values for these complexes encapsulated in mesoporous silicas with larger pore size. The SOD activity was measured by the Beauchamps and Fridovich indirect assay using the NBT reagent,<sup>33</sup> in phosphate buffer of pH 7.8. Previously, the stability of the encapsulated complexes in this medium was verified by UV-vis spectroscopy after incubation of at least 1 h, at room temperature, longer than the time-length of the SOD's test. Different batches of each hybrid material suspended in phosphate buffer of pH 7.8 were prepared, sonicated and then centrifuged prior to the spectrophotometric measurements. In the case of 1@MSSP, release of the tricationic complex 1<sup>3+</sup> was negligible (Figure S5, left), while for 2@MSSP, 10-12% leakage of the complex from the silica matrix was evidenced (Figure S5, right), probably due to the weaker interaction of this complex with the silica surface.

The NBT indirect assay is based on kinetic competition between NBT and the catalyst for the in situ photogenerated superoxide, and the SOD activity is inversely related to the amount of formazan formed by reaction of NBT with superoxide, observed at 560 nm. Blank experiments with unloaded MSSP particles corroborated that the silica matrix had no effect on NBT reduction. As shown in Figure S6, the two hybrid materials inhibit the reduction of NBT, and the  $IC_{50}$ values (the concentration of the catalyst that lowers by 50% the NBT reduction) of 0.033 µM (1@MSSP) and 1.46 µM (2@MSSP) were determined from the plots of % inhibition vs [catalyst]. These values were used to calculate the second-order McCord-Fridovich rate constants,  $k_{MCF} = k_{NBT} [NBT]/IC_{50}$ , which are independent of the concentration and type of detector.<sup>34</sup> The  $k_{MCF}$  values for complexes 1 and 2, free and immobilized, are listed in Table 2. It is evident that encapsulation enhances de reactivity of 1 and this effect is more pronounced as the pore size of the silica matrix decreases (entries 2-5 in Table 2). This result reinforces the hypothesis that a pore diameter close to the complex size avoids the separation of the [(dien)Cu]<sup>+</sup> and [HImCu(dien)]<sup>2+</sup> moieties after reaction with O<sub>2</sub><sup>•-</sup>, preserving its activity as in the native enzyme (eq. 1 and 2). In this way, when [(dien)Cu]<sup>+</sup> reacts with O<sub>2</sub><sup>•-</sup>, proton transfer from the neighbor [HImCu(dien)]<sup>2+</sup> fragment assists the substrate reduction, and enhances the complex efficiency.

$$\begin{split} & [N_{3}Cu(II)ImCu(II)N_{3}] + H^{+} + O_{2}^{\bullet-} \rightarrow [N_{3}Cu(II)ImH + Cu(I)N_{3}] + O_{2} \quad (1) \\ & [N_{3}Cu(II)ImH + Cu(I)N_{3}] + H^{+} + O_{2}^{\bullet-} \rightarrow [N_{3}Cu(II)ImCu(II)N_{3}] + H_{2}O_{2}(2) \end{split}$$

Another factor affecting the SOD activity is the distortion degree of the Cu(II) center, because a more distorted environment is best suited to accommodate the reduced Cu(I) form of the catalyst.<sup>35,36</sup> The *f*-factor ( $g_{//}$  /  $A_{//}$  ratio) value calculated from EPR parameters can be used as a measure of the tetrahedral distortion from the  $N_4$ -square planar geometry around Cu(II), and varies from 105 to 135 cm

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-like activity of free and immobilized complexes									
	Catalyst	∱(cm)	k <sub>мс</sub> (М⁻¹ s⁻¹)	Pore size	Reference				
1	CuZn-SOD (native)	160	200 x 10 <sup>7</sup>		37,41				
2	1		0.78 x 10 <sup>7</sup>		18				
3	1@MSSP	128	8.28 x 10 <sup>7</sup>	2.7	This work				
4	<b>1</b> @MCM-41	117	5.15 ×107	3.4	17				
5	<b>1</b> @SBA-15	120	2.35 ×107	8.6	16				
6	2	-	0.85 x 10 <sup>6</sup>		18				
7	<b>2</b> @MSSP	-	1.87 x 10 <sup>6</sup>	2.7	This work				
8	<b>2</b> @MCM-41	-	0.59×10 <sup>6</sup>	3.4	17				
9	<b>2</b> @SBA-15	-	0.456×10 <sup>6</sup>	8.6	16				
10	3@MSN-FITC	163	6.53 x 10 <sup>7a</sup>	2.6	38				
11	<b>4</b> @Al-MCM-41-N⁺	138	15.6 x 10 <sup>7</sup>	2.5	39				
12	4@silica gel	113 <sup>b</sup>	1.65 x 10 <sup>6a</sup>	6	40				

 $\mathbf{3} = [(bipyridinyl)_2Cu - \mu - pbi-Zn(pbi)](ClO_4)_2$ , pbi = 2-(2-pyridyl)-benzimidazole. FITC = fluorescein isothiocyanate, MSN = mesoporous silica nanoparticles.  $\mathbf{4} = [(dien)Cu(\mu - Im)Zn(tren)]^{3+}$ , tren = tris(2-aminoethyl)amine.

<sup>a</sup> Calculated from reported data using  $k_{\text{NBT}}$  (pH = 7.8) = 5.94 × 10<sup>4</sup> M<sup>-1</sup> s<sup>-1.42</sup> <sup>b</sup> Calculated from reported g<sub>//</sub> and A<sub>//</sub> values.

for square planar complexes and  $\approx$  250 cm for tetrahedral complexes.<sup>31</sup> The f-factor for free and encapsulated complex **1** are given in Table 2. The value of f = 128 cm for 1@MSSP indicates the Cu(II) site of the complex encapsulated in MSSP is more distorted than in mesoporous silicas with wider pores (f-factor = 117-120),<sup>16,17</sup> although less distorted than the local environment of Cu(II) in the native enzyme (f-factor = 160).<sup>37</sup> Therefore, the higher constraints imposed to the complex by the small channels of MSSP also favor the SOD activity of complex 1. A suitable explanation is that steric constraints can control the redox potential of the Cu(II)/Cu(I) couple, inasmuch as the small channel can better accommodate the tetrahedral geometry preferred by Cu(I).<sup>36</sup> Therefore, inside the pore, the reduced Cu(I) complex should be stabilized and the redox potential shifted towards less negative values, favoring the SOD activity. The same order of reactivity with O2. was observed for two pyridylbenzimidazolate- and imidazolate-bridged dinuclear catalysts,<sup>38,39</sup> complexes 3 and 4 of entries 10-11 in Table 2, encapsulated in mesoporous matrices with similar channels diameter and higher metal local environment distortion. In contrast, when complex 4 was encapsulated in a silica matrix with larger pore size (entry 12), the copper center was less distorted and the SOD activity decreases significantly.40

Ultimately, the reduced pore size allows the two mononuclear fragments to remain close to each other to react with O<sub>2</sub><sup>•-</sup>, as well as induces a higher distortion of the ligand around the metal center, and both effects favor the SOD activity of the imidazolate-bridged dimetal complex. In the case of complex **2**, encapsulation in MSSP also improves the reactivity compared to the complex free and encapsulated in MSPs with wider pores (entries 6-9 in Table 2), probably because the small pore width forces the complex to adopt a constrained and more reactive conformation.

#### 4. Conclusions

Complexes **1** and **2** were successfully inserted into the silica matrix of MSSPs with pore width of 2.7 nm, retaining the morphological and textural properties of the guest-free silica particles. The small silica channels constrain the geometry of the complexes enhancing the distortion of the local Cu(II) environment to a larger extent than in mesoporous silicas with wider pores. The hybrid materials are robust and stable. The strong electrostatic interactions between tricationic **1** and the surface groups on pores are a decisive factor for the full retention of the complex within the silica matrix, while **2** is partially located at the pores mouth. The increased catalytic SOD activity shown by **1@**MSSP and **2@**MSSP as compared to the same complexes free and encapsulated in mesoporous silicas with larger pore sizes highlights the close fitting of the pore diameter to the catalyst dimensions is a key factor to improve their reactivity.

#### **Author Contributions**

Conceptualization, S.R.S. and V. D.; Methodology, Resources, S. R. S., V. D. and N. P.; Funding Acquisition, S. R. S. and V. D.; Investigation, M. P. and M. L.; Formal analysis M. P., M. L., S. R. S., V. D. and N. P.; Writing – Original Draft Preparation, S. R. S.; Writing – Review & Editing, S. R. S., V. D. and N. P.; Supervision: V. D.

#### **Conflicts of interest**

There are no conflicts to declare.

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