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SYNTHESIS AND STUDY OF SOD MIMETICS: FROM AZA-MACROCYCLIC COMPLEXES TO NANO-STRUCTURED SYSTEMS

The dismutation of superoxide radical anions is a key metabolic process to prevent oxidative damage. Inspired by the natural enzymes such as superoxide dismutases (SOD), we have designed a new set of aza-macrocycles, which form binuclear Cu^{2+} complexes, as shown by potentiometric and spectroscopic studies. The binuclear Cu^{2+} complexes show significant SOD activity, which is remarkably increased when the macrocycles are grafted onto boehmite nanoparticles (BNPs). The observed increase can be ascribed to the positive ζ-potential of the BNPs.

Oxygen plays a key role in maintaining the vital functions of the organism, especially in those related with the energy metabolism. Indeed, oxygen consumption is particularly large in the neurological tissues, where neurones and astrocytes use it in order to carry out the cellular respiration, as well as in the biosynthesis of neurotransmitters. This is why, although comprising 2% of the total body weight, the brain is responsible of around 20% of total body oxygen consumption. But this can also explain why brain is particularly susceptible to oxidative damage.

When the redox-active metal ions present in the organism (such as Cu^{2+} or Fe^{2+}) are unregulated, they can lead to an excess production of reactive oxygen species (ROS) *via* Fenton chemical reactions. Thus, the disruption of the metal-ion regulatory pathways may result in the aggregation of the proteins or even in the generation of free radicals and ROS. If the amount of ROS cannot be assumed by the scavenging systems of the organism, it gives rise to oxidative stress [1].

The imbalance between the generation and the clearance of the reactive oxygen species is related to a variety of health issues, such as cardiovascular diseases or diabetes. But, since the brain is an organ especially susceptible to the oxidative damage, some of the most important disorders caused by ROS are neurological. Some of the most well-known are Alzheimer's, Parkinson's and Huntington's diseases. In Alzheimer's disease, for instance, Cu and Fe contribute to aggregate the beta-amyloid plaques in the affected regions of the hippocampus, which play a vital role in increasing levels of oxidative injury [2].

In order to remove ROS, living organisms have developed a battery of protective enzymes, such as superoxide dismutases (SODs), catalases and peroxidases. Mammalian SODs contain either CuZn-binuclear centres (SOD1 and SOD3) or Mn mononuclear centres (MnSOD, SOD2). But, although treatment with natural enzymes such as SOD, Vitamin E or coenzyme Q10 has been tested, and they were found to be neuroprotective, no proven benefit was shown in the clinical settings. It can be explained attending to the severe drawbacks that therapy with natural enzymes presents, such as the absence of oral activity, immunogenicity, short half-life and low cell permeability [1, 3]. These results are indicative of the need for low-molecular weight mimetics, containing both ionophore and antioxidant activity: small molecules

capable of bimodal modulation of the metal-ions. Previous studies have shown that a number of these low molecular SOD mimetics are complexes of polyamine ligands of either cyclic or open-chain topology [4-6].

Given the success of such mimetics, we designed a new set of aza-macrocycles (see Scheme 1) not only with the capacity to disrupt the metal-induced beta-amyloid plaque formation by chelation of free metals in solution, but also with antioxidant and protective activity against ROS. Furthermore, these ligands were also functionalised in order to go a step forward in the design of new SOD mimetics: a hydroxo group was rightly disposed in the structure of the hexaaza-macrocycles to permit its covalent anchorage to boehmite (γ-AlO(OH)) nanoparticles (BNPs). Since non-toxic nanoparticles (NPs) may improve the activity, the likely-cell uptake and bio-distribution of these low molecular weight mimetics, grafting of the macrocycles onto their surface might be a good way to take advantage of the profits that nanoscience provides [7, 8].

Regarding the synthesis of the macrocycles, they were prepared using a modification of the Richman-Atkins procedure, reacting the pyridine derivative 4-benzyloxy-2,6-bis(bromomethyl)pyridine with different pertosylated polyamines (1,5,9,12,16,20-hexakis(ρ-tolylsulfonyl)-1,5,9,12,16,20-hexaazaicosane for **L1** and 1,5,9,13,17,21- hexakis(ρ-tolylsulfonyl)-1,5,9,13,17,21-hexaazaheneicosane for **L2**). The removal of the tosyl and benzyl groups was performed using HBr/AcOH with an excess of phenol. Finally, grafting of the ligands onto the oxidic nanoparticles was performed by condensation of the pyridinol groups of the ligand with the terminal Al-OH groups at the surface of the nanoparticles. The amount of anchored macrocycle was quantified by elemental microanalysis and ^{1}H NMR calibration using TSP as internal standard. The concentration of the ligands onto the surface is around 10^{-4} mol per gram of nanoparticle ($[\text{L1}] = (3.5\pm0.4) \cdot 10^{-5}$ mol/g_{BNP} and $[\text{L2}] = (2.20\pm0.02) \cdot 10^{4}$ mol/g_{BNP}).

Previously to the antioxidant activity studies, we proceeded to characterise the Cu^{2+} complexation behaviour of the ligands in solution. Thus, by using
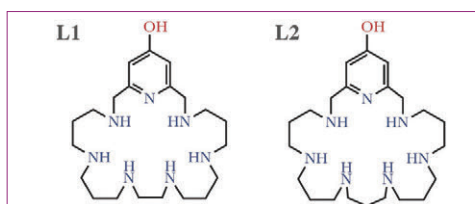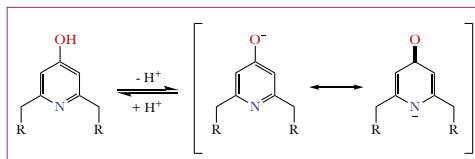

Scheme 1 - Drawing of the ligands studied in this work

Fig. 1 - Representation of the keto-enolic equilibrium of the deprotonated pyridinol moiety

potentiometric techniques, as well as by measuring the UV-Vis spectrum of solutions of the ligands in function of the pH, we first determined the protonation constants of the macrocycles. Both ligands show seven protonation constants ranging from 10.68 to 4.14 logarithmic units and corresponding to the six secondary amines and the phenol group of the pyridine derivative. It is worth noting that pyridinol moiety deprotonates at a particular acidic pH (*ca*. 5~6). It can be explained attending to the keto-enolic equilibrium shown by the non-protonated form of the pyridinol (see Fig. 1), which allows the formation of salt bridges between the negatively charged nitrogen of the pyridine moiety and the protonated amines of the macrocycle, as shown by DFT modelling studies.

pH-metric speciation studies of the systems Cu^{2+}-**L1** and Cu^{2+}-**L2** at variable pH show the formation of mononuclear $[\text{CuH}_x(\text{H}_{-1}\text{L})]^{(2+x)+}$ species with x ranging from 0 to 5, as well as the binuclear species of $[\text{Cu}_2\text{H}(\text{H}_{-1}\text{L})]^{4+}$, $[\text{Cu}_2(\text{H}_{-1}\text{L})]^{3+}$, $[\text{Cu}_2(\text{H}_{-1}\text{L})(\text{OH})]^{2+}$ and $[\text{Cu}_2(\text{H}_{-1}\text{L})(\text{OH})_2]^+$ stoichiometries. The distribution diagrams collected in Fig. 2 show that for a mole ratio Cu^{2+}:L 2:1 the binuclear species prevail above pH 3 for **L1** and above pH 6 for **L2**. It is interesting to notice that the deprotonation of the hydroxyl group of the pyridinol takes place as soon as the macrocycle coordinates the first Cu^{2+} atom. This suggests that the pyridinol nitrogen is always involved in the complexation of the copper atoms. The large values of the stability constants found for the complex $[\text{Cu}(\text{H}_{-1}\text{L})]^+$ (23.56 logarithmic units for **L1** and 19.25 for **L2**) leads to deduce that besides the pyridinol nitrogen, other four amines coordinate simultaneously the metallic atom. So, the addition of a second Cu^{2+} is accompanied by much lower stability constants (8.80 logarithmic units for **L1** and 6.95 for **L2**) that can be ascribed to a lower number of nitrogen atoms participating in its coordination. Moreover, some bond breaking and reorganisation might accompany this second step. The unsaturated coordination sphere of the second metal ion and consequent reduced stability of the Cu^{2+} complex at this stage should lead to a better cycling between oxidation states Cu(II) and Cu(I) required for an efficient SOD activity to occur.
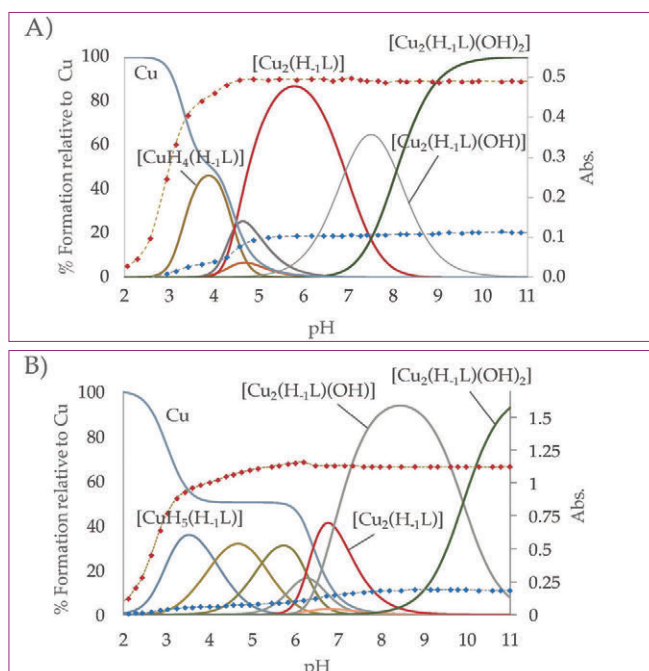
Fig. 2 - Distribution diagram of the Cu^{2+}:L systems for the ligands A) **L1** and B) **L2** as a function of the pH in aqueous solution ([Cu^{2+}] = 2·10^{-3} M; [L] = 10^{-3} M). The UV-Vis spectroscopic parameters of the pyridine system (red dots, λ = 285 nm for the Cu^{2+}:**L1** system and 281 nm for Cu^{2+}:**L2**) and d-d transition band (blue dots, λ = 590 nm for the Cu^{2+}:**L1** system and 606 nm for Cu^{2+}:**L2**) are overlaid

Once the Cu^{2+} complexes in solution were characterised, we carried out the antioxidant activity assays by using the McCord-Fridovich method [9, 10]. This is based on an indirect assay in which the side production rate of superoxide anions by the enzyme xanthine oxidase is determined by measuring the reduction rate of the dye nitroblue tetrazolium to give formazan. A compound displaying SOD activity will decrease the flow of superoxide radical anions and thereby, the production of formazan. Blank experiments were recorded with the ligands alone, as well as the BNPs with and without the ligands grafted onto its surface, without observing any effect.

The values of k$_{cat}$ collected in Tab. 1 allow deriving several conclusions. First, all the Cu^{2+} complexes present a remarkable SOD activity, particularly the binuclear ones. It can be explained attending to the unsaturated coordination sphere of the metallic atoms in the binuclear complexes,

System	IC$_{50}$ (µM)	kcat (10^6 M^{-1} s^1)
Cu-L1	1.4(5)[a]	2.5
Cu$_2$-L1	0.8(1)	4.1
Cu$_2$-L2	0.37(4)	9.3
Cu$_2$-BNP-L1	0.10(3)	33.7
Cu$_2$-BNP-L2	0.12(1)	28.7
Cu(ClO$_4$)$_2$[b]	1.1(1)	2.7
CuZn-SOD[b]	0.010(2)	430

a) Values in parenthesis are standard deviations in the last significant figure. b) Taken from ref. [11]

Tab. 1 - Evaluation of the SOD activity of the Cu^{2+} systems with **L1** and **L2** at pH = 7.4

as above commented. This leads to a lower stabilisation of the Cu^{2+} complexes, favouring the cycling between Cu^{2+} and Cu$^+$ required for promoting the dismutation of the superoxide anions.

Second, grafting of the ligands onto the BNPs leads to a very significant enhancement of the Cu^{2+} complexes SOD activity, up to an 8-fold increase in the case of Cu$_2$-**L1**. This can be related to the pre-concentration of Cu^{2+} complexes on the surface of the BNPs, as previously described [11, 12]. However, since superoxide radicals are negatively charged, the positive surface of the BNPs (see Tab. 1) could contribute to the SOD activity enhancement by attracting the superoxide anions to the Cu^{2+} active centres, favouring the catalytic process to occur. This process somehow evokes the active centre of the CuZn-SOD enzyme, which presents a funnel with a gradient of increasing positive charge as the redox-active metal is approached.

In order to check the effect of the surface charge on the catalytic activity, negatively charged silica nanoparticles were prepared and grafted with **L1** and **L2**. Thus we obtained a set of NPs functionalised with Cu$_2$-**L1**, which show a negative charge (-12(2) mV), and another set of NPs functionalised with Cu$_2$-**L2**, which show neutral charge (0(2) mV). The different charge of the nanoparticles can be ascribed to the concentration of ligand onto its surface, which rapidly increases the surface charge of the system, as well as to the acid-base behaviour of the anchored macrocycles. Thus, the greater the amount of grafted ligand, the more positive the charge of the NPs is (see Tab. 2).

The SOD activity results for these nanosystems, collected in Tab. 2 and Fig. 3, show that negatively charged nanoparticles decrease the catalytic constant of the Cu^{2+} complexes, while neutral NPs barely have any effect on their SOD activity. Therefore, the results support that the electrostatic interaction between the surface of the NPs and the superoxide anionic substrates has a key role in the SOD activity increases or decreases exerted by the nanosystems.

Conclusions

The binuclear Cu^{2+} complexes of two pyridinol hexaaza-macrocycles have shown to be extremely efficient

System	IC50 (μM)	k_{cat} (10^6 M^{-1} s^{-1})	[L] (mol_L g_{np}^{-1})	ζ-potential (mV)
Cu$_2$-**BNP-L1**	0.10(3)[a]	33.7	0.35(4)·10^{-4}	24.7(1)
Cu$_2$-**BNP-L2**	0.12(1)	28.7	2.21(4)·10^{-4}	32.0(7)
Cu$_2$-**SNP-L1**	3.5(3)	1.0	0.23(2)·10^{-4}	-12(2)
Cu2-**SNP-L2**	0.45(3)	8.2	0.33(3)·10^{-4}	0(2)
Cu(ClO$_4$)$_2$[b]	1.1(1)	2.7	-	-
CuZn-SOD[b]	0.010(2)	430	-	-

a) Values in parenthesis are standard deviations in the last significant figure.
b) Taken from ref. **[11]**

Tab. 2 - Evaluation of the SOD activity and charge of the surface (ζ-potential) of the Cu^{2+} systems with L1 and L2 when grafted to the boehmite and silica nanoparticles at pH = 7.4

in enhancing the dismutation rate of superoxide anions, that can be related to the unsaturated coordination sphere of the copper in such complexes. Grafting of the macrocyclic systems onto the surface of boehmite nanoparticles show a drastic increase in the SOD activity of the complexes, enhancement that can be mainly explained attending to the positive charge of the nanoparticles that would help driving the anionic substrates to the active centre. Studies performed with silica nanoparticles, which depending on the grafted molecules, show negative or close to zero ζ-potentials exhibit either decrease or similar activities to the free binuclear complexes.

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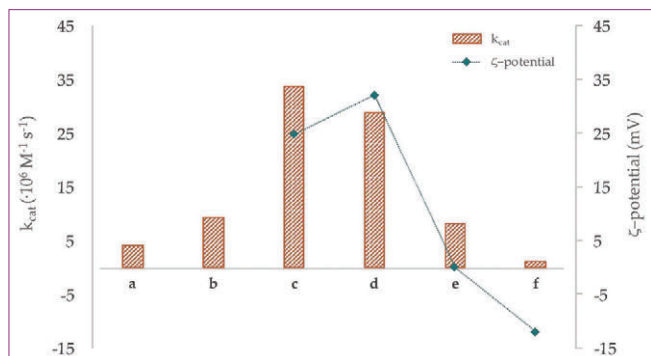
Fig. 3 - Representation of the catalytic constant (orange bars) and ζ-potential (blue dots) values of the systems a) Cu$_2$-L1, b) Cu$_2$-L2, c) Cu$_2$-BNP-L1, d) Cu$_2$-BNP-L2, e) Cu$_2$-SNP-L2 and f) Cu$_2$-SNP-L1

Sintesi e studio di composti mimetici di SOD: dai complessi aza-macrociclici a sistemi nanostrutturati

La dismutazione dei radicali-anioni superossido è un processo metabolico chiave per prevenire il danno ossidativo. Traendo ispirazione da enzimi naturali come le superossido dismutasi (SOD), abbiamo progettato una nuova serie di aza-macrocicli, che formano complessi binucleari di Cu^{2+}, come dimostrato da studi potenziometrici e spettroscopici. Questi complessi binucleari di Cu^{2+} mostrano una significativa attività SOD, che aumenta notevolmente quando i macrocicli vengono innestati su nanoparticelle di boehmite (BNP). L'aumento osservato può essere attribuito al potenziale-ζ positivo delle BNP.