

**RESEARCH ARTICLE****NOVEL ARTIFICIAL SUPEROXIDE DISMUTASE (SOD) BASED ON  $\text{Cu}^{\text{II}}$ (SAL-ALA)/MGALLDH  
AND  $\text{Cu}^{\text{II}}$ (SAL-PHEN)/MGALLDH HYBRIDS****MIHAELA MURE EANU<sup>1\*</sup>, CRISTINA B BEANU<sup>1</sup>, LIVIA ELENA BIBIRE<sup>2</sup>, GABRIELA CÂRJ <sup>2\*</sup>**

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**Abstract**

Novel hybrids with superoxide scavenging activity were obtained from the complexes Cu(II)-Schiff base derived from salicylaldehyde and aminoacids (L-alanine and L-phenylalanine) immobilized onto MgAILDH support. The properties and superoxide dismutase activity of the Cu(II) complexes/LDH biomimetic hybrids were investigated. The EDX, XRD, FTIR DRUV XPS and TG/DTA results show the formation of a new hybrid layered materials by physical adsorption of the complexes onto the LDHs inorganic matrix besides other weaker interactions. This novel artificial enzymes possess very good superoxide dismutase activity and the advantages of heterogeneous catalysts as easy recovery and repeat utilization.

**Keywords:**  $\text{Cu}^{\text{II}}$  complexes, salicylidene-amino acid Schiff base, MgAILDH, Superoxide dismutase activity

**Introduction**

Our cells rely on oxygen as the final acceptor of electrons in respiration, but oxygen could be also a dangerous compound. The formation of reactive forms of oxygen, such as superoxide (oxygen with an extraelectron) in aerobic organisms can cause disintegration of cell membranes, damaging protein and DNA structures and is linked to aging, cancer, and neurodegenerative diseases (Perry et al., 2007). To combat this potential danger, most cells make superoxide dismutase (SOD). The natural SOD enzymes are a class of metalloenzymes which contain Cu/Zn, Fe, or Mn complex at the active site and catalyze this dismutation of the free radical superoxide, but some major drawbacks associated with instability, denaturation in the reaction conditions have halted the application of SOD as a therapeutic agent (Tang et al., 2002).

Some of the synthetic metal complexes have shown to possess favorable SOD activity and promising clinical effects and have been studied in order to be developed into pharmaceutical candidates (Salvemini et al., 2002; Riley, 1999).

Furthermore, the immobilized complexes, often called bioinspired catalysts because their activities and selectivity may resemble to those of the enzymes, are capable of working under more rigorous conditions and they can easily be recovered and recycled (Kervinen et al., 2006). In this biomimetic catalysts the central ion is a redox-active transition metal ion and the ligands are amino acids or other molecules having groups that are able to coordinate to it (Silva et al., 2013). Superoxide dismutase enzyme mimicking Cu(II)

and Cu(II)–Zn(II) complexes were immobilized on silica gel by adsorption (Szilágyi et al., 2005a), on montmorillonite through electrostatic forces (Szilágyi et al., 2005b) or were grafted on different type of silica by ionic interactions (Fang et al., 2011). Cu(II)–histidine complexes were covalently grafted onto a chlorinated polystyrene resin as Cu,ZnSOD enzyme mimics (Szilágyi et al., 2009).

Mesoporous clay matrices, and especially layered double hydroxides (LDHs), are interesting to be used as supports in complex catalytic systems as they possess a unique combination of intercalation, swelling and exchange characteristics, which endows their ability to host on their surfaces or in the interlayer spaces various cationic species or complexes (Rives and Ulibarri, 1999; Wang, Q. and O'Hare, 2012; Wang, M.-Z. et al., 2013; Wang, X. et al., 2012). It was proved that Mg/Al-NO<sub>3</sub>-LDH matrix protect the cancer pharmaceuticals based on Cu<sup>2+</sup> complexes of salicylidene-amino acids Schiff bases, in solution, especially in acid environment (Wang, M.-Z. et al., 2013).

We have recently presented two Cu(II)-Schiff base complexes immobilized onto LDH supports as novel catalytic systems for the cyclohexene oxidation by H<sub>2</sub>O<sub>2</sub> (Mureseanu et al., 2014). In order to extend the application area of these new hybrid materials we have developed a novel biomimetic catalyst. Consequently, in this work we present the facile synthesis of the Cu(II) complexes with Schiff base ligands derived from salicylaldehyde (Sal) and L-alanine (Ala) or L-phenylalanine (Phen) aminoacids. The Cu<sup>II</sup> Sal-Ala and Cu<sup>II</sup> Sal-Phen complexes as active center and the MgAlLDHs as solid support were used to establish a novel artificial SOD. The activities of the complexes were compared and the SOD activity was demonstrated. From our knowledge, this highly active and selective biomimetic catalysts are presented for the first time. Furthermore, these new hybrid materials can be easily immobilized to fabricate biomimetic sensor or other catalytic systems and can solve many practical problems such as easy recovery, repeat utilization and resistance in extreme conditions.

## Experimental

### Materials

All chemicals were commercially purchased and used without further purification. Mg(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O, Al(NO<sub>3</sub>)<sub>3</sub>·9H<sub>2</sub>O, Cu(CH<sub>3</sub>COO)<sub>2</sub>·H<sub>2</sub>O, Na<sub>2</sub>CO<sub>3</sub>,

NaOH, salicylaldehyde, L-Alanine, L-Phenylalanine (Sigma–Aldrich) for the immobilized complexes synthesis, riboflavin, L-methionine and nitro blue tetrazolium (NBT) for the biochemical test of superoxide dismutase activity were used in this study. Solvents such as methanol, ethanol, and acetonitrile were purchased from Merck and used without further purification.

## Synthesis procedures

### Preparation of LDH

The parent LDH was prepared by the pH controlled co-precipitation of the corresponding metal nitrate salts, followed by an ageing step of the synthesis medium at 45°C for 24h. Typically, an aqueous solution of 0.075 mol/L Mg(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O and 0.025 mol/L Al(NO<sub>3</sub>)<sub>3</sub>·9H<sub>2</sub>O was added dropwise to 100 mL of deionized water at 65°C under vigorous magnetic stirring. The pH of the mixture was kept at a constant value equal to 9 ± 0.1 by simultaneous addition of an aqueous solution containing 0.1 mol/L Na<sub>2</sub>CO<sub>3</sub> and 0.5 mol/L NaOH. After ageing, the resulting slurry was filtered, washed thoroughly with deionized water till the washings were neutral and dried at 100°C overnight.

### Synthesis of the metal complexes

The Cu<sup>II</sup> complexes were synthesized as described in literature (Sakiyan et al., 2001) and denoted Cu<sup>II</sup>(Sal-Ala) or Cu<sup>II</sup>(Sal-Phen). Alanine or phenylalanine (10 mmols) was added into a methanolic solution (50 mL) of NaOH (20 mmols). Salicylaldehyde (10 mmols) in 50 mL methanol and the copper acetate (5 mmols) were added and the mixture was kept under continuous stirring for 3h at RT. The volume was reduced to 1/4 of the initial value, the solid was filtered and recrystallized from a mixture of methanol-ethanol (2:1).

### Cu<sup>II</sup> Complexes/LDHs biomimetic catalysts

LDH was dried under reduced pressure at 100°C. Fifty milliliters of absolute ethanol was added to 1 g dried LDH and stirred for 30 min. The ethanolic suspension of LDH with 0.5 mmols of metal complex was transferred and refluxed for 24h with constant stirring and under nitrogen atmosphere. The final products (denoted Cu<sup>II</sup>(Sal-Ala)/LDH and Cu<sup>II</sup>(Sal-Phen)/LDH) were isolated by filtration, washed with bidistilled water, then with acetonitrile and kept overnight in vacuum at 60°C.

### Physical-chemical characterization

Powder X-ray diffraction (XRD) measurements were performed on a Bruker AXS D8 diffractometer by using Cu K $\alpha$  radiation ( $\lambda = 0.154$  nm) over a  $2\theta$  range from 3 to 70 degree. The FTIR spectra were recorded using a Bruker Alpha spectrometer. The UV-Vis diffuse reflectance spectra were recorded using a Thermo Scientific (Evolution 600) spectrometer. The copper content was determined by flame atomic absorption spectrometry (AAS) on a Spectra AA-220 Varian Spectrometer with an air-acetylene flame. C, H, and N contents were evaluated by combustion on a Fisons EA1108 elemental analysis apparatus. Thermogravimetric analysis (TG/DTA) was carried out in a Netzsch TG 209C thermobalance in nitrogen flow. The XPS spectra were obtained with a X-ray spectroscopy (ULVAC-PHI).

### Catalysis of superoxide dismutation

Each free or immobilized Cu(II) complexes as well as the MgAILDH support were tested for SOD activity using the Beachamp-Fridovich reaction (Beauchamp and Fridovich, 1971). The SOD activity of the biomimetic catalysts was assayed by measuring the inhibition of the photoreduction of NBT. This indirect assay consists of several reactions: riboflavin is photochemically excited and reduced thereafter by methionine into a semiquinone, which donated an electron to oxygen to form the superoxide anion. The superoxide readily converted NBT into a purple formazon product. The transformation can be followed by spectrophotometry measuring the absorbance at 560 nm. The addition of the SOD enzyme or a SOD-mimicking material inhibits formazon formation. The quantity of enzyme inhibiting the reaction by 50% ( $IC_{50}$ ) is defined as one unit of SOD (Misra and Fridovich, 1972). In this regard, the lower the  $IC_{50}$  value, the higher the SOD activity is. The SOD activity measurement was carried out at room temperature in a suspension of immobilized complex at pH = 7 ensured with a phosphate buffer. For the free complex samples a methanolic solution containing the same amount of copper as the immobilized samples was used. The reaction mixture contained 0.1 mL of 0.2 mM riboflavin, 0.1 mL of 5 mM NBT, 2.8 mL of 50 mM phosphate buffer with the L-methionine (13 mM) and the catalyst. Riboflavin was added last and the reaction was initiated by illuminating the tube with a 30 W fluorescent lamp. Equilibrium could be reached in

15 min. The inhibition curve of NBT photoreduction by increasing the concentration of free or immobilized complex were constructed in order to determine the  $IC_{50}$  value (in  $\mu$ M) for each sample. We have used samples without catalysts to give a background visible absorbance value.

Native SOD (7.46 U) from bovine erythrocytes was used as positive control.

## Results and Discussion

### Characterization of Cu<sup>II</sup> Complexes/LDHs biomimetic catalytic systems

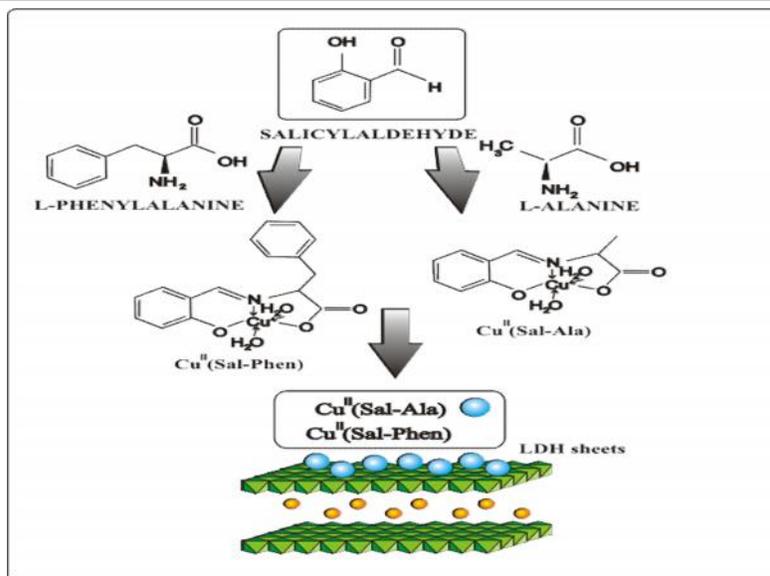
#### Elemental analysis

Two Schiff base ligands derived from salicylaldehyde and alanine or phenylalanine amino acids and their Cu<sup>II</sup> complexes have been synthesized and immobilized thereafter on the MgAILDH support (Scheme 1).

Elemental analysis results confirm the composition of the complexes and of the hybrid LDHs (Table 1). This analysis indicates that all of the complexes are monomeric, being formed by the coordination of 1 mol of metal and 1 mol of Schiff base ligand. The greater amount of N% for the hybrid composites could be explained by the presence of nitrate anions into the interlayer space of LDH even after the complexes immobilization, as the TG analysis have confirmed (Fig.3).

#### Powder X-ray diffraction

Powder X-ray diffraction diagrams (XRD) of both immobilized Cu<sup>II</sup> complexes and the LDH matrix (Fig. 1) are quite similar and exhibit some common features, such as narrow, symmetric, strong peaks at low  $2\theta$  values and weaker, less symmetric lines at high  $2\theta$  values. For layered hydroxide-like materials these peaks (0.74, 0.37 and 0.26 nm) correspond to diffraction by planes (003), (006), and (009), respectively. Hence, the overall structure of LDH is preserved upon the Cu<sup>II</sup>(Sal-Ala/Phen) immobilization and is clear that newly-formed hybrid composites are of Cu<sup>II</sup>(Sal-Ala/Phen)/MgAILDH type. Almost all the introduced quantity of copper complex (0.5 mmol/g) is anchored to the solid (as indicated by AAS analysis and Table 1) and one can propose that the copper(II) complexes are immobilized on the surface or at the edges and/or defects of the crystal surface as indicated (Parida et al., 2010) for related systems.

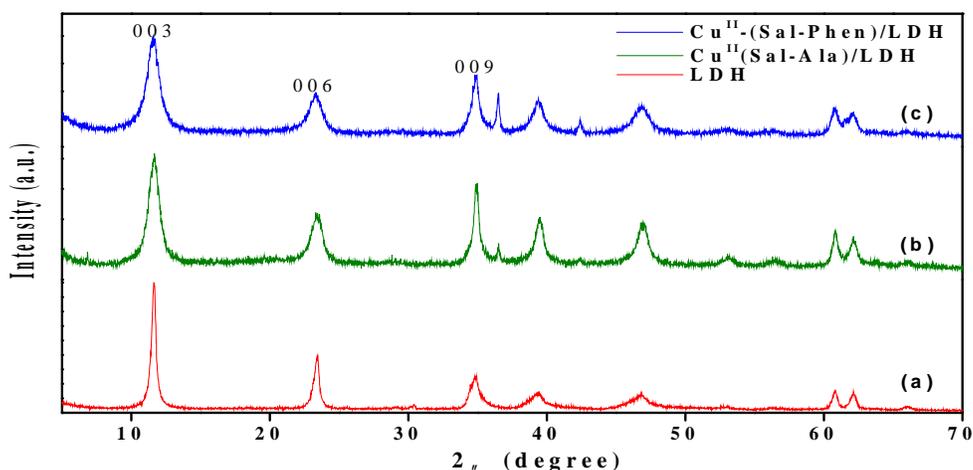


**Scheme 1.** Cu<sup>II</sup>(Sal-Ala/Phen)/LDH biomimetic catalytic systems

**Table 1.** Elemental analysis of Cu<sup>II</sup>-Schiff base complexes free or LDH-supported

Compound	Analytical data <sup>a</sup> (%)				Cu/N molar ratio	Immobilization yield, %
	C	H	N	Cu		
Cu <sup>II</sup> (Sal-Ala), C <sub>10</sub> H <sub>13</sub> NO <sub>5</sub> Cu	42.31 (41.21)	4.63 (4.47)	4.52 (4.80)	21.72 (21.80)	1/1	-
Cu <sup>II</sup> (Sal-Ala)/LDH	6.66 (5.46)	1.86 (0.59)	3.58 (0.69)	2.86	1/6	90
Cu <sup>II</sup> (Sal-Phen), C <sub>16</sub> H <sub>18</sub> NO <sub>5</sub> Cu	52.65 (52.29)	4.98 (4.92)	3.62 (3.79)	17.28 (17.31)	1/1	-
Cu <sup>II</sup> (Sal-Phen)/LDH	7.16 (5.76)	1.90 (0.54)	3.90 (0.42)	1.91	1/9	60

<sup>a</sup> Calculated values are shown in parenthesis; for the immobilized complexes, the C%, H% and N% were calculated only for the ligand corresponding to the Cu% determined by AAS, considering a metal to ligand ratio of 1/1.



**Figure 1.** XRD patterns of (a) LDH, (b) Cu<sup>II</sup>(Sal-Ala)/LDH and (c) Cu<sup>II</sup>(Sal-Phen)/LDH

### FTIR, diffuse reflectance UV-Vis and XPS analyses

The coordination environment around Cu<sup>II</sup> and the process of the complexes immobilization were investigated using FTIR, UV-Vis and XPS analyses. Fourier transform infrared (FTIR) spectra of hybrids (Fig. 2(a)) display the characteristic bands of both pristine LDH and complexes. Thus, (C=N), <sub>as</sub>(COO<sup>-</sup>), <sub>s</sub>(COO<sup>-</sup>), (Cu-O) and (Cu-N) were all present in the FTIR spectra of the homogeneous complexes, and the band positions agree well with published data (Wang et al., 2010). Furthermore, the complex spectra contain new bands in the 800-600 cm<sup>-1</sup> region that could be attributed to the (Cu-O) (phenolic oxygen), 701-500 cm<sup>-1</sup>, to (Cu-O) (carboxylic oxygen) and 500-600 cm<sup>-1</sup> respectively, for the (Cu-N) valence vibration. The band at 3450cm<sup>-1</sup> may be due to some uncoordinated -OH groups of phenyl ring. For the LDH support, the broad absorption between 3600 and 3300 cm<sup>-1</sup> is due to the (OH) mode of the hydroxyl groups, both from the brucite-like layers and from interlayer water molecules. Interlayer water also gives rise to medium-intensity δ(H<sub>2</sub>O) absorption close to 1628 cm<sup>-1</sup>. The band at 1333 cm<sup>-1</sup> is assigned to the stretching vibration of interlayer NO<sub>3</sub><sup>-</sup>. The bands at wavenumber lower than 850 cm<sup>-1</sup> are due to the M-O and O-M-O vibrations of the hydroxalcite (Parida et al., 2010). In the FTIR spectra of the immobilized complexes, apart from the bands in the overlapping regions of the LDH support, only the <sub>as</sub>(COO<sup>-</sup>) band is clearly present at 1563 cm<sup>-1</sup> (see Fig. 2). However, compared with their corresponding homogeneous complexes, the different positions of the (COO<sup>-</sup>) could be the consequence of this group involvement in the complexes immobilization on the LDH surface (Mac Leoda et al., 2012).

The UV-visible diffuse reflectance(DRUV) spectra of free and immobilized complexes are shown in Fig. 2(b). The UV-Vis spectra of the homogeneous complexes all displayed three typical peaks: at 250 nm due to benzenoid - \* transition, 380 nm assigned to a ligand-to-metal charge-transfer transition and the third peak around 600 nm associated with a *d-d* transition. The UV-Vis spectrum of Cu<sup>II</sup>(Sal-Ala/Phen)/MgAILDH showed similar features to the free complexes and to each other, indicating that during immobilization no change of the Cu<sup>II</sup> coordination center took place. However, the intensity of *d-d* transition band was lower due to the small amount of complex immobilized onto the LDH support.

The Cu2p XPS spectra (Fig. 2(c)) shown a main line at 932.9 eV that reflect a charge transfer metal 3d<sup>10</sup> to ligand L<sup>-1</sup>(Kawai et al. 1994). The electronic state of divalent copper compound is expressed by a resonance of ionic and covalent state. XPS data confirms the presences of copper only as complex on the surface of LDH support.

### Thermogravimetric analysis

The thermal stability of MgAILDH support and of the Cu<sup>II</sup>(Sal-Ala/Phen)/MgAILDH hybrid composites was studied and the thermogravimetric (TG) curves are presented in fig. 3.

Generally, four steps are observed in the thermal evolution of LDHs. The first two steps correspond to the removal of physically adsorbed and intergallery water (30-100°C, 2.37% mass loss) and the dehydroxilation of the brucite-like layers (100-250°C, 13.69% mass loss). The third weight loss (250-500°C, 24.99% mass loss) is due to the decomposition of the nitrate anions. The last step (500-900°C, 4.57% mass loss) correspond to the LDH complete decomposition with oxide formation. The thermal decomposition of Cu<sup>II</sup>-complexes/LDH proceeds in four distinct steps similar to each other and to that of the LDH, but the DTA maxima of their thermal effects are shifted by 10-50°C (first step), 2-20°C (second step) and 50°C (third step) comparatively with the LDH matrix.

For Cu<sup>II</sup> (Sal-Ala)/LDH, the first step (30-150°C, 3.83% mass loss) correspond to the removal of physisorbed and interlayer water and the second one (150-252°C, 11.25 % mass loss) to the partial elimination of structural hydroxyl groups in the basic layers. The sharp mass loss observed in the range 250-600°C (29.20% mass loss) is due to the total dehydroxylation of the host layers, the decomposition of the organic guests and of the nitrate anions presents in LDH interlayers. There is a fourth mass loss step in the range 600-900 °C (2.10% mass loss).

Cu<sup>II</sup>(Sal-Phen)/LDH present the first two step between 30-230°C (14.46% mass loss) that correspond to water and structural hydroxyl removal, ligand and nitrate anions decomposition (230-600°C, 31.80% mass loss) and the complete thermal decomposition with oxide formation, between 600-900°C, mass loss of 2.21%.

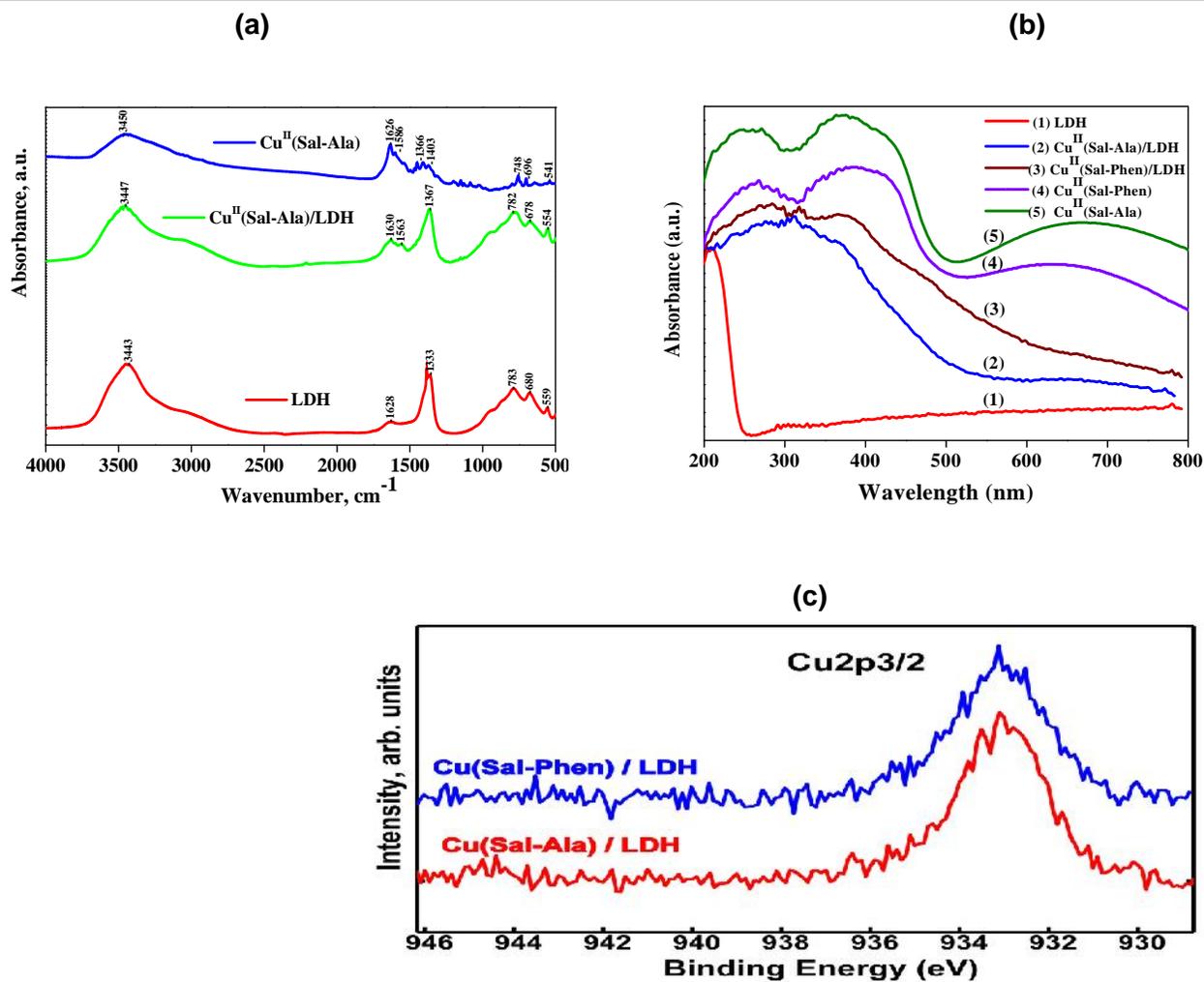


Fig. 2. FT-IR (a), UV-Vis (b) and high resolution XPS spectra of  $\text{Cu}2p_{3/2}$  (c)

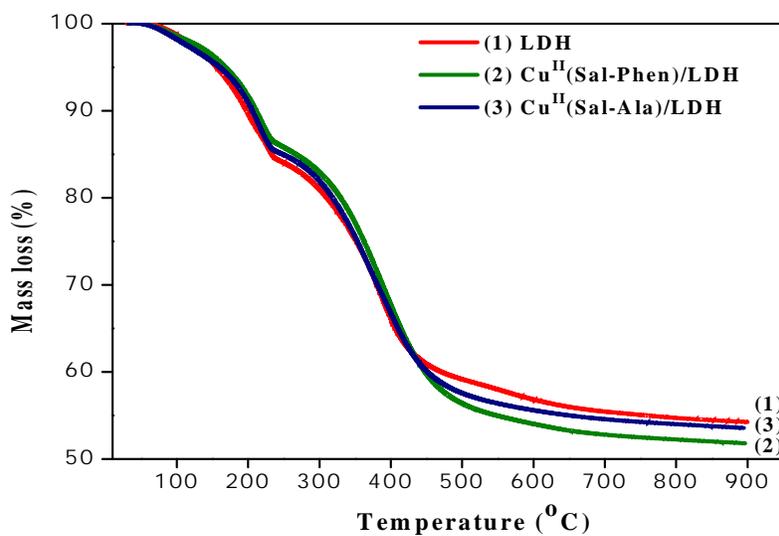
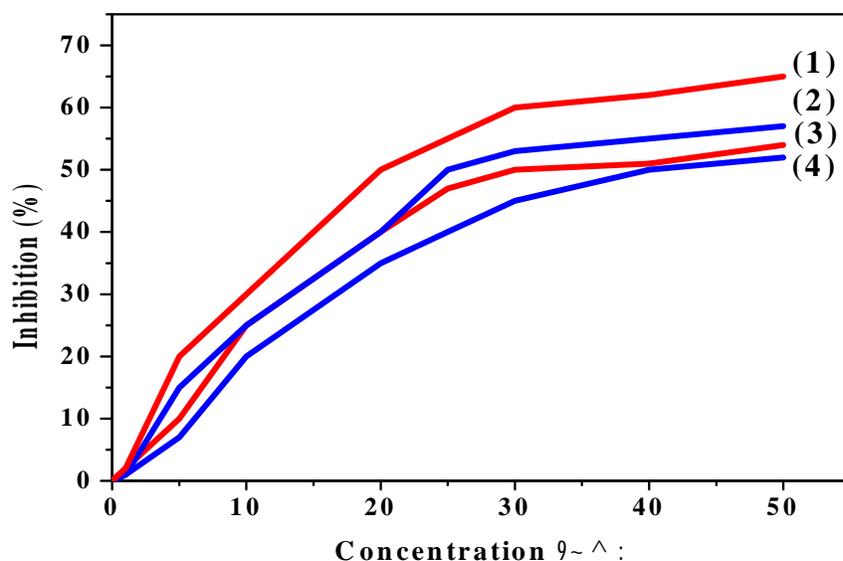


Fig 3. TG curves of (a) LDH, (b)  $\text{Cu}^{\text{II}}(\text{Sal-Ala})/\text{LDH}$ , (c)  $\text{Cu}^{\text{II}}(\text{Sal-Phen})/\text{LDH}$



**Fig. 4.** Inhibition of NBT photoreduction by increasing concentration of (1) Cu<sup>II</sup> (Sal-Ala)/LDH; (2) Cu<sup>II</sup> (Sal-Phen)/LDH; (3) Cu<sup>II</sup> (Sal-Ala); (4) Cu<sup>II</sup> (Sal-Phen)

The similar thermal behaviour of LDH support and of the hybrid composites and the presence of the nitrate anions in the LDH interlayer space after the copper complexes immobilization prove that the complexes are not intercalated into this space. Furthermore, the mass losses calculated from TG curves are in accordance with the greater %C and %N determined by the elemental analysis.

Considered together, the EDX, XRD, FTIR, DRUV, XPS and TG/DTA results show the formation of a new hybrid layered material by physical adsorption of the amino acid Schiff base complexes onto the LDHs inorganic matrix, beside other weaker interactions such as hydrogen bonds, electrostatic interactions or donor-acceptor interactions between the complexes and the surface -OH groups (Mac Leoda et al. 2006; Wang et al. 2013).

#### Superoxide dismutase (SOD) activity of Cu<sup>II</sup> Complexes/LDHs hybrids

Each Cu(II) complex free or immobilized onto the MgAlLDH support, as well as the clay without complexes were tested for SOD activity by measuring inhibition of the reduction of NBT. All materials, excepting the LDH support, displayed catalytic activity in the dismutation reaction of superoxide radical anions and their activity is comparable and even better than other SOD biomimetic copper complexes immobilized into different supports (Szilágyi et al., 2005a, Szilágyi et al. 2005b; Fang et al., 2011; Szilágyi et al., 2009).

The inhibition (%) of different artificial SOD concentration was calculated and the curve is shown in Fig. 4. For the free Cu<sup>II</sup> (Sal-Ala) and Cu<sup>II</sup> (Sal-Phen) complexes the IC<sub>50</sub> values were 30 µM and 37 µM respectively. On immobilization the SOD activity has changed. The IC<sub>50</sub> values became Cu<sup>II</sup> (Sal-Ala)/LDH: 20 µM and Cu<sup>II</sup> (Sal-Phen)/LDH: 25 µM. For the immobilized complexes the SOD activities increased. Probably the LDH matrix protect the complex that mimics the active centre of the natural SOD enzyme and could enhance its catalytic activity by a more favorable environment, more easy accessibility of the substrate and high dispersion of the activity centers. Compared with complexes, these novel artificial enzyme systems can be easily immobilized into different supports by different techniques in order to obtain many devices such as packed column, film devices, carriers of biomolecules and medicines, etc. Furthermore their activity will be retained due to the protection of rigid-solid structure of LDHs that makes them easier recovered and reused than the complexes.

#### Conclusion

Novel artificial superoxide dismutase (SOD) based on Cu<sup>II</sup>(Sal-Ala/Phen) complexes supported on MgAlLDH matrices were prepared and tested in the dismutation reaction of superoxide radical anions. The immobilization of Cu(II)-based complexes on the LDH support beneficially contributed to the increasing SOD activity.

This rigid artificial SOD present not only functions of SOD but also advantages over other low molecular weight biomimetic complexes, that is, this hybrids with superoxide scavenging activity can be easily immobilized to fabricate biomimetic catalytic unit and they can be easily recovered for repeat utilization. Potential application of this new hybrid materials for protection, transport and controlled release of Cu(II) complexes with Schiff bases derived from aminoacids is under consideration.

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