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Antibacterial activity of copper monodispersed nanoparticles into sepiolite

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Abstract Copper monodispersed nanoparticles (2–5 nm) embedded into submicron particles of sepiolite ($Mg_8Si_{12}O_{30}(OH)_4(H_2O)_4{\cdot}8H_2O$), suitable to be used for biological applications have been obtained after a specific treatment and subsequent reduction process. Cu/Sepiolite particles have revealed as a strong bactericide (similar to Triclosan) so that they were able to decrease the starting microorganism concentrations of *Staphylococcus aureus* or *Escherichia coli* by 99.9%.

Introduction

Nowadays, nanotechnology is playing an important role in the field of health. In this sense, nanoparticles are being used in biomedical [1, 2] and biological [3] applications. Indeed, it is well known that some kind of metallic nanoparticle, such as silver, copper and zinc, have antibacterial capabilities [4]. In order to facilitate the nanoparticle handling and to reduce health risks, nanoparticles in inorganic matrix are being studied as antibacterial agents [5]. These materials present high antibacterial activity, low toxicity, chemical stability, long lasting action period and thermal resistance versus organic antibacterial agents. Due to the stability of these nanoparticles supported on a matrix, they can be used in paint or plaster as a bactericide agent [6] for coat hospital equipment, as well as fittings for public places, public transport, paints, toys and kitchen, school and hospital utensils.

In the present work, we have prepared copper monodispersed nanoparticles into submicron needle-like particles of a magnesium phylosilicate called sepiolite ($Mg_8Si_{12}O_{30}(OH)_4(H_2O)_4{\cdot}8H_2O$). The structure of the sepiolite is as a quincunx arrangement of talc-type layers separated by parallel channel [7]. It has been reported that sepiolite has a very reduced ion exchange capacity but the largest specific surface (300 m^2/g) between all the phylosilicates. However, after acid treatment we were been able to exchange some proportion of the magnesium ions located at octahedral positions by copper ions. Afterwards, by means of a reduction process of the interchanged cations linked to a crystal structure folding copper monodisperse nanoparticles appear into the matrix.

The antibacterial effect of copper nanoparticles into sepiolite was tested by two different microorganisms a Gram-positive bacterium (*Staphylococcus aureus*) and a Gram-negative bacterium (*Escherichia coli)*. There is nowadays a worldwide concern due to the serious social problem caused by harmful microorganisms such as legionella infections, hospital infections caused by medicine-resistant bacteria such as MRSA (methicillin-resistant *S. aureus*) [8, 9] and food poisoning caused by *S. aureus*.

The main advantage of this kind of materials is the large quantity of metallic nanoparticles that can be easily obtained. Additionally, as a consequence of the fact that nanoparticles are supported on silicate

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microparticles manipulation becomes easier and stability against oxidizing atmosphere increase remarkably. Therefore, the nanoparticles manipulation health risks, recently reported [10, 11], are considerably reduced. Furthermore, these nanoparticles have been obtained by a simple low cost procedure and can be easily scaled-up for industrial applications.

Table 1 Microorganisms optimal growth conditions

Microorganism	Medium	Incubation	
		Time (h)	*T*ª (K)
S. aureus	Agar nutrient (AN)	24	300
E. coli	Agar nutrient (AN)	24	300

Experimental methods

Copper monodispersed nanoparticles into sepiolite were obtained starting from sepiolite powder (TOLSA) purified and micronized by a wet process with a particle size below 1 μm. Sepiolite powder was dispersed at 10 wt.% concentration in water using high shear mixing and then the suspension was acidified with HNO_3. Afterwards, 1.5 l of this sepiolite suspension was mixed with 1.0 l of aqueous solution of Cu $(NO_3)_2 \cdot 6H_2O$, so that the final relative metal concentration into sepiolite was 12 wt.%. Thereafter, the pH of the dispersion was adjusted with NaOH in order to precipitate the metallic cations. Then, the dispersion was vacuum filtered and washed with water. The resulting powder was reduced in a 90% Ar/10% H_2 atmosphere at 500 °C for 2 h to obtain finally copper nanoparticles.

X-ray diffraction patterns were recorded in a Bruker D8 diffractometer using CuKα radiation. The weight loss and structural changes during thermal decomposition of samples were determined by thermal gravimetric analysis (TGA) and differential thermal analysis (DTA) curves in a Stanton Mod. STA 781. Transmission Electron Microscopy (TEM) images were taken by a JEOL microscope model FXII operating at 200 kV.

The antibacterial activity of copper nanoparticles into sepiolite was tested following the method "antimicrobial products-test for antimicrobial activity and efficacy" according to the Japanese Industrial Standard JIS Z 2801:2000. The microorganism: *E. coli* (CECT 516) and *S. aureus* (CECT 239) were used for the test. The bacterial suspensions were prepared by cultivating the bacteria for 18 h with agitation in 0.2 vol.% Broth Nutrient solution. Each microorganism was reconstituted with 2 ml of 0.2 vol.% Broth Nutrient solution and incubated for optimal growth conditions (Table 1). Then, the solutions were diluted with 0.2 vol.% sterile Ringer solution establish the bacterial concentration in the range from 2.5×10^5 to 10×10^5 (CFU)/ml.

Antimicrobial activity was measured by the addition of 9 ml of 0.2 vol.% sterile Ringer solution to the Cu/Sepiolite sample so that the copper concentration was 1 wt%. Afterwards, 1 ml of the inoculum solutions were added and incubated (see Table 1). The solutions were diluted and collected on a dish to count the numbers of viable cells through a 24 h period.

A lixiviation study of Cu/Sepiolite has been performed in order to know the copper concentration that sepiolite is able to release. Cu/Sepiolite was dispersed in ultra pure water so that the copper concentration was 1 wt% at 37 °C for 1, 5 and 24 h. Suspensions were filtered and copper concentration of the resulting solutions were quantitatively analysed by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES), using a Thermo Jarrell Ash model Iris Advantage spectrophotometer.

Result and discussion

The mechanism of nanoparticle formation is as following: the Mg^{2+} cations can be interchanged by some other metal in an acid solution [12–14] ($pH < 2$).

$$2(SiO){-}Mg + 2\,H^+ \leftrightarrows Mg^{2+} + 2\,SiOH \qquad (1)$$

Subsequently, increase of the pH solution by the addition of NaOH definitely fixes metallic substitutes into octahedral crystallographic position [15, 16] according to:

$$n\,SiOH + M^{n+} \leftrightarrows n(SiO){-}M + n\,H^+ \qquad (2)$$

The most remarkable feature of this silicate is that after a reduction process, metallic cations reduce destabilizing the crystallographic silicate structure. As a result, as far as nanoparticles form, silicate structure covers them, providing protection against coarsening and oxidation. In fact, the novelty of this method is that nanoparticles are not deposited on a passive substrate, but both, nanoparticles and substrate are formed together.

The XRD patterns of sepiolite treated with $Cu_2(OH)_3NO_3$ prior and after the reduction process are shown in Fig. 1. As it can be seen in this figure, after a detailed analysis of diffractogram, the $Cu_2(OH)_3NO_3$ phase is detected (Fig. 1b) by XRD. In addition, XRD patterns of as received and anhydrous sepiolite were included for comparation purpose in Fig. 1. In the case of Cu/Sepiolite after the reduction process, only the Cu peaks are present (Fig. 1d).

The thermal evolution in air of pure Mg-sepiolite presents the loss of zeolitic water and a double dehydration process corresponding to the loss of two pairs of coordination water molecules [17]. A similar behaviour can be found in the case of Cu/Sepiolite (Fig. 2a). However, the thermal evolution in hydrogen atmosphere (Fig. 2b) presents a double dehydration process corresponding to the loss of zeolitic water at 75 °C, and a second step corresponding to the loss of the coordination water and reduction of the copper cations between 204 and 365 °C. In both Figs. 2a and b, we show thermal decomposition (endothermic peak at 240 °C) and reduction process (exothermic peak at 270 °C) of $Cu_2(OH)_3NO_3$ precursor, which has not been substituted by the magnesium cations leached from sepiolite. It should be noted that in this mineral, magnesium and substituted copper cations appear disposed in planes of octahedral coordinated atoms. These polyhedra hold the silicate structure, so that, if they disappear due to the reduction process, the sepiolite structure collapses. As a result, octahedral polyhedra disappear to form small copper nanoparticles (~5 nm) and the sepiolite structure collapses over them avoiding coalescence process.

The TEM micrographs of Cu/Sepiolite are shown in Fig. 3. As observed copper metallic nanoparticles are monodispersed and located along the sepiolite particles with a narrow size distribution. The size distribution reaches its maximum between 2 and 5 nm.

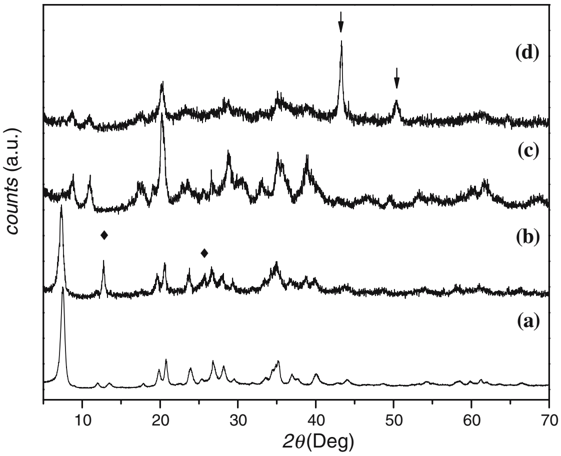

Fig. 1 XRD powder patterns corresponding to Sepiolite (**a**), Cu/Sepiolite (diamonds indicate $Cu_2(OH)_3NO_3$ peaks) (**b**), reduced Sepiolite (**c**) and reduced Cu/Sepiolite (arrows indicate Cu peaks) (**d**)

Antimicrobial Test for antimicrobial activity has revealed that copper nanoparticles are an excellent antibacterial agent on Gram-positive and Gram-negative bacteria. Table 2 shows its antibacterial effect by *S. aureus* and *E coli* microorganism. The numbers of viable cells from inoculum control and substance were used to calculate the reduction to the microorganism Eq. (3) and logarithm reduction rate (log η) Eq. (4):

$$24\text{h} - \text{reduction}(\%) = [(A - B)/A] * 100 \tag{3}$$

$$\text{Log}\ \eta = \log A - \log B \tag{4}$$

where A is the average number of viable cells from inoculum control after 24 h and B is the average number of viable cells from substance after 24 h. Antibacterial activity has been compared with Triclosan [18, 19] (chlorinated aromatic), a commercial broad-spectrum antibacterial/antimicrobial agent. The results reveal that the efficacy of Cu/Sepiolite is identical to that of Triclosan as seen in Table 2. No antibacterial activity was found when pure sepiolite was tested.

Although the biocide effect of copper is well known [4] we interpret that the high effectiveness of the Cu/Sepiolite system is the result of the combination of the effect of copper spreaded along the sepiolite and the large specific surface of sepiolite. In fact, it can be stated that sepiolite acts as an effective scaffold, where copper nanoparticles are perfectly monodispersed. As the antibacterial activity of copper depends on as much as free surface on liberate ions, the role of sepiolite avoiding agglomeration of copper nanoparticles is crucial. Then, it can be concluded that in this particular case, sepiolite acts as an accessible surface enhancer of copper nanoparticles. This is not the general trend, in a recent publication, Top and Ülkü [20] have shown that Cu^{2+} into zeolite have almost negligible antibacterial activity.

Lixiviation study of Cu/Sepiolite showed that the copper concentration in water increased from 1 to 5 h. Instead the concentration decreased for the sample at 24 h (Table 3). The pH value of the silicates after the different tests were found to be ~10. Then the decreasing in copper concentration is likely due to the precipitation of $Cu(OH)_2$ along the test at this particular pH. In any case, the obtained copper concentration in solution is high enough to ensure it excellent antibacterial activity [21]. Some other interesting result is that XRD pattern of the dried powder of the Cu/Sepiolite sample used on this study presented no

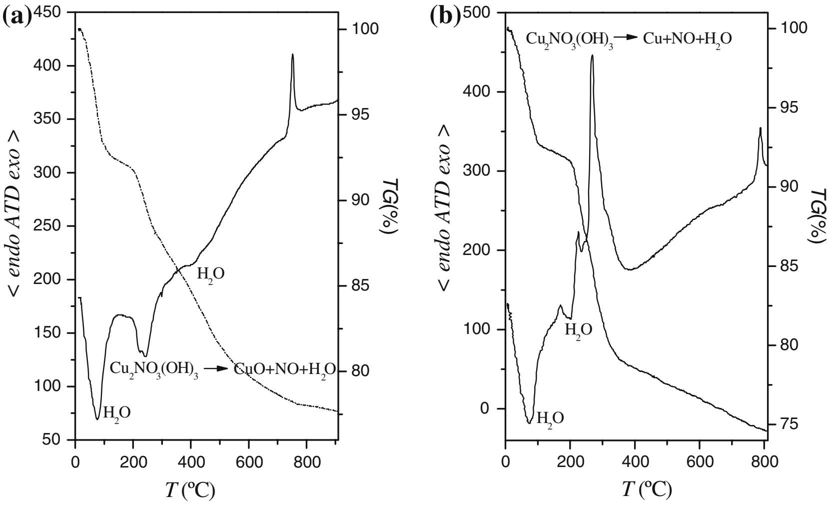

Fig. 2 (**a**) ATD/TG in air atmosphere of Cu substituted Sepiolite; (**b**) ATD/TG of the same sample in hydrogen atmosphere

appreciable reduction of metallic copper. It seems to indicate that sepiolite plays an important role by delaying the liberation of metallic copper in solution.

It should be noted that all characterizations were carried out in air, several weeks after the preparation of the samples. This means that nanoparticles are protected against oxidation by the sepiolite matrix.

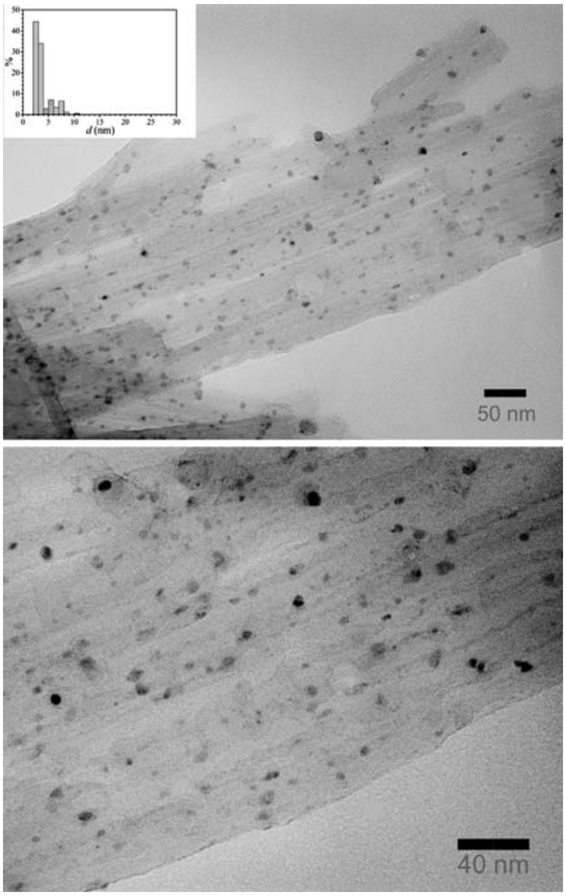

Fig. 3 TEM micrographs corresponding to Cu/Sepiolite with their corresponding metal particle size distributions (insets) at two different magnifications

Conclusions

Copper monodispersed nanoparticles with a narrow size distribution (2–5 nm) have been obtained into sepiolite following a simple low cost wet chemical route. The fact that these nanoparticles appear supported on an inert matrix makes easy its manipulation and enhances its biocide activity. It has been proved that copper nanoparticles into sepiolite reduce the *S. aureus* and *E. coli* concentrations by 99.9%, achieving a safe disinfection.

According to the results, we can conclude that stable copper metallic can be easily obtained and scaled-up to produce large amounts of commercial nanoparticles (tonnes) embedded into sepiolite.

Table 2 Antibacterial effect of copper metal nanoparticles into sepiolite

Microorganism	24 h-reduction (%)		Log reduction	
	SepCu	Triclosan	SepCu	Triclosan
S. aureus	99.99	99.99	4.70	4.31
E. coli	99.99	99.99	4.40	4.55

Table 3 Copper concentrations lixiviated in water for 1, 5 and 24 h of test at 37 °C

	Lixiviated 1 h (37 °C)	Lixiviated 5 h (37 °C)	Lixiviated 24 h (37 °C)
Cu (ppm)	5.55	6.33	0.19

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