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Improved antimicrobial activity of silica–Cu using a heteropolyacid and different precursors by sol–gel: synthesis and characterization

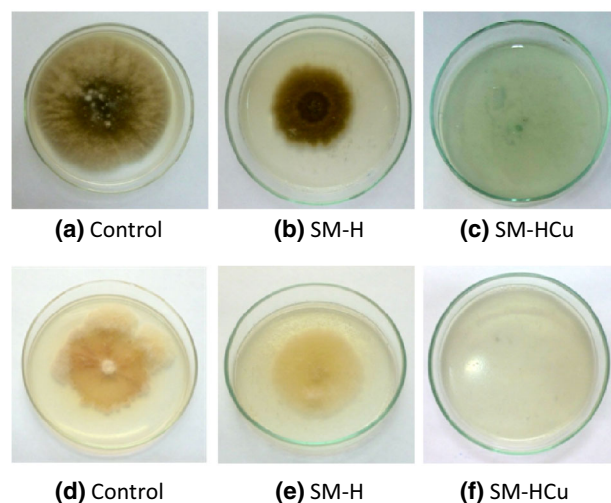
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Abstract Antimicrobial coatings are used to avoid the proliferation of fungi and bacteria inside urban buildings by incorporating low concentrations of antimicrobial agents (biocides). This study is focused on evaluating the antifungal activity of copper (II) supported on a silica matrix, and as counter-cation of a heteropolyacid, obtained from the sol–gel method using various catalysts, against two fungal isolates. The fungi used in this evaluation were *Alternaria alternata* and *Chaetomium globosum* isolated by routine microbiological techniques from biodeteriorated paints films. In all cases, fungi were selected due to their ability to grow on the paint films and their negative impact on human health. First, silicas were prepared using tetraethylorthosilicate and methyl trimethoxysilane as precursors and acetic acid, hydrochloric acid as catalysts of the hydrolysis reaction of the alkoxide. The effect of addition of copper (II) in the form of copper nitrate was studied. With respect to the morphology of the silica, most of them are similar, between pure and modified silica, in all cases. Once the stage of the synthesis and characterization of the prepared materials was performed, six of them were selected to evaluate their antifungal activity by agar plate inhibition test against both fungi of interest (*A. alternata* and *C. globosum*), and the percent inhibition was

determined in each case. Tested Cu-based solids were shown to have a higher antifungal activity because they completely inhibit the growth of both fungi with lower concentrations relative to its control. This work was performed as a preliminary study, in order to guide the selection of a suitable organic biocide from a list of possible antifungal agents.

Graphical Abstract



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Keywords Antimicrobial coatings · Silica · Copper · Sol–gel method · Antifungal activity

1 Introduction

There is a global concern about microbial colonization, especially on clinical and food industrial surfaces [1, 2]. It is well known that the microorganisms attach to diverse substrates, multiply and embed in the extracellular polymeric

substance matrix they produce [3, 4]. This development, called biofilm, provides a higher resistance of the comprised microorganisms to hydrodynamic shear forces, biocides or antibiotics [4, 5]. Therefore, prevention of microorganisms colonization is clearly necessary, especially fungal proliferation which recently was strongly associated with infections, respiratory symptoms, allergies, asthma and immunological reactions [6, 7]. On the other hand, they are also involved in the deterioration and degradation of both synthetic and natural materials, secreting enzymes and organic acids during their metabolic process [8]. Among the microorganisms that colonize the indoor environment, fungi are considered the most damaging [9, 10].

The development of new materials or coatings might prevent microbial colonization, but to make this possible they must contain bioactive agents (called biocides) [5, 11], usually present in low concentrations as additives [11]. At the present time, the tendency in the antimicrobial formulation is to replace the traditional biocides with environmentally friendly ones [12].

These kinds of coatings are obtained by adding metals (e.g., silver or copper), germicides (e.g., triclosan), natural bioactive compounds (e.g., peptides) or by other methods (e.g., light-activated antimicrobials, bioactive polymers [1, 13]). Silica matrix has several advantages such as: high-purity precursors, homogeneity of the chemically inert, stable and homogeneously obtained material and can be designed to act as a reservoir for releasing antimicrobial agents over extended time periods [14, 15].

Recent works have focused on the antifungal efficiency of different particulate metals [16, 17]. The antimicrobial properties of copper, silver, titanium and zinc are effective in the reduction in diverse microorganisms growth [18–21].

The Green chemistry main target is to make new chemical processes or change the existing ones so that they will be more benign for the environment. This is why the need to develop clean technologies rises. A sustainable development is obtained when new technologies are provided to society to cover their needs but with responsibility toward the environment [22, 23].

The main goal of this work is the synthesis and characterization of mixed materials based on silica prepared by the sol–gel method, using tetraethylorthosilicate (TEOS) and methyltrimethoxysilane (MTMS) as precursors; acetic acid, hydrochloric acid and a heteropolyacid (HPA) as hydrolysis catalysts. Also, this HPA was incorporated along with copper nitrate ((NO₃)₂Cu) as active agent, to assess its inhibiting activity against two fungi isolated from an indoor biodeteriorated substrate [24], *Alternaria alternata* and *Chaetomium globosum*, for future application as biocide in antimicrobial coatings.

The synthesis of these silicas represents an environment-friendly alternative, in concordance with the trend of the new antimicrobial formulation to replace traditional biocides. The fungi were selected by their ability to grow and deteriorate

different materials, especially biofilms, in addition to the negative effect that they have on human health [25, 26].

2 Experimental section

2.1 Materials

The materials tetraethylorthosilicate, also named tetraethoxysilane, (Si(OCH₃)₄) (TEOS), and methyltrimethoxysilane (CH₃Si(OCH₃)₃) (MTMS) were used as silica precursors; acetic acid (99.99 %) (AcH), hydrochloric acid (99.99 %) (HCl), and molybdophosphoric acid (H₃[P(Mo₃O₁₀)₄] \cdot xH₂O) (HPA) as catalysts; absolute ethanol (CH₂CH₃OH, 99.9 %) (EtOH) and distilled water as solvents in all the synthesis. (NO₃)₂Cu was added in the necessary quantity.

The sol–gel method was used to synthesize the solids based on silica, under nitrogen atmosphere. Twelve samples were obtained using tetraethoxysilane (Si(OCH₃)₄) (TEOS) and methyltrimethoxysilane (CH₃Si(OCH₃)₃) (MTMS) as silica precursors. In order to obtain different structures of siliceous gels, acetic acid (99.99 %) (AcH), hydrochloric acid (99.99 %) (HCl) and molybdophosphoric acid (H₃[P(Mo₃O₁₀)₄] \cdot xH₂O) (HPA) were used as catalysts, whereas absolute ethanol (CH₂CH₃OH, 99.9 %) (EtOH) and distilled water were used as solvents in all the synthesis.

In the synthesis with acetic and hydrochloric acids, the molar ratios of the reagents were 1:1.17:5:3.73 for precursor/catalyst/solvent/water, respectively. The amount of each component was obtained from previous assays of the research group to obtain, approximately, 10 g of silica. In the samples prepared using HPA, this reagent was added in order to reach a final concentration in the solid of 11 % (w/w) and (NO₃)₂Cu was added in the proper amount to occupy the half of the acid sites of HPA (H_{1.5}Cu_{1.5}).

To obtain the different solids, a portion of solvent, specific catalyst, alkoxide precursor and copper were incorporated and put into a beaker, and the addition of the remaining amount of ethanol was then carried out. Only in the synthesis with HCl, the catalyst was added at the end of the procedure. Subsequently, a slow addition of water was carried out and the mixture was stirred for 2 h. Finally, the wet gel particles were dried at room temperature and atmospheric pressure up to complete solidification. The reagents and the nomenclature for all the samples are shown in Table 1.

2.2 Characterization of the solids

2.2.1 Potentiometric titration with *n*-butylamine

The evaluation of the acidic properties of solids was achieved by potentiometric titration with *n*-butylamine, carried out in a Metrohm 794 Basic Titrino titrator

Table 1 Composition and nomenclature used for the prepared samples

Sample	Precursor	Catalyst	Active phase
ST-HAc	TEOS	AcH	Cu(NO ₃) ₂
ST-HCl	TEOS	HCl	
ST-H	TEOS	HPA	
ST-HCu	TEOS	HPA	
SM-HAc	MTMS	AcH	Cu(NO ₃) ₂
SM-HCl	MTMS	HCl	
SM-H	MTMS	HPA	
SM-HCu	MTMS	HPA	
STM-HAc	TEOS-MTMS	AcH	Cu(NO ₃) ₂
STM-HCl	TEOS-MTMS	HCl	
STM-H	TEOS-MTMS	HPA	
STM-HCu	TEOS-MTMS	HPA	

(Switzerland), with a double-junction electrode. An *n*-butylamine solution in acetonitrile (0.025 N) was added, at a 0.025 mL/min rate, to 0.025 g of sample, previously suspended in acetonitrile (45 mL), and stirred for 3 h.

2.2.2 Textural properties

The textural properties of the silicas, such as the specific surface area (S_{BET}), were determined in Micromeritics Accusorb 2100 equipment (USA), using N₂ as absorbable gas.

2.2.3 X-ray diffraction

The X-ray diffraction (XRD) patterns were obtained in Philips (Holland) PW-1390 (channel control) and PW-1394 (motor control) equipment coupled to a scanning graphical recorder, using Cu K α ($\lambda = 1.5417 \text{ \AA}$) radiation, Ni filter, 20 mA and 40 kV in the voltage source, a 5–60 2 θ scanning angle range, a scanning rate of 2°/min and 2000 counts/s for the amplitude of the vertical scale.

2.2.4 Scanning electron microscopy

Scanning electron microscopy (SEM) was used to obtain different micrographs of the solids, in Philips 505 equipment (Holland), using a voltage of 15 kV. Samples were supported on graphite and metallized with a sputtered gold film. The micrographs were obtained with an ADDAII acquisition device (Soft Imaging System).

2.2.5 FT-IR

FT-IR spectra were obtained using Bruker IFS 66 equipment and pellets of the sample in KBr, measured in a range between 400 and 4000 cm⁻¹.

2.2.6 Antimicrobial activity

Fungi, *A. alternata* and *C. globosum*, previously isolated from biodeteriorated surfaces [27] were used as bioindicators to evaluate the antifungal activity of the silicas with HPA inside (control) and with HPA-Cu. Sub-cultures were obtained into Petri dishes from the isolates previously mentioned. The solid culture medium (CM-agar) was composed of agar, 1.5 g; dextrosa, 1 g; peptone, 0.5 g; KH₂PO₄, 0.1 g; MgSO₄·7H₂O, 0.05 g; and distilled water.

The solids with inhibitory activity were evaluated in vitro as fungal growth inhibition on agar plates [24, 25] with different concentrations against the fungal growth (*A. alternata* and *C. globosum*). Spores were removed from the sub-cultures prepared previously and deposited in a flask (5 mL) of physiological solution containing NaCl [0.85 % (w/v)] and Tween 20 [0.0005 % (w/v)], in order to obtain a suspension. The concentration of spores was adjusted to 0.3–0.5 × 10⁶ spores/mL employing a Neubauer chamber.

Plates with 15 mL of CM-agar were prepared with different concentrations of the studied solids (0.1, 0.5 and 1.0 mg/mL in relation to Cu content), as well as the control (without copper) and the other without the addition of any agent (only CM-agar). The CM-agar plates were inoculated with 20 μ L of each fungal suspension in a single point located at the center and incubated at 25 °C. After 10 days, the average diameter of fungal colony was recorded and the inhibition effect was calculated according to the following equation [25]:

$$I = [(C - E)/C] \times 100 \quad (1)$$

where *I* is inhibition (%), *C* and *E* are the average colony diameter of the mycelium growth of the controls and the evaluated solid plates, respectively. The data refer to the results of the last day of growth, applying three parallel measurements to obtain the standard deviations [25]. Through these data, the minimum inhibitory concentration (MIC), which was defined as the lowest tested concentration where no fungal growth was observed, is obtained.

3 Results and discussion

3.1 Characterization of the solids

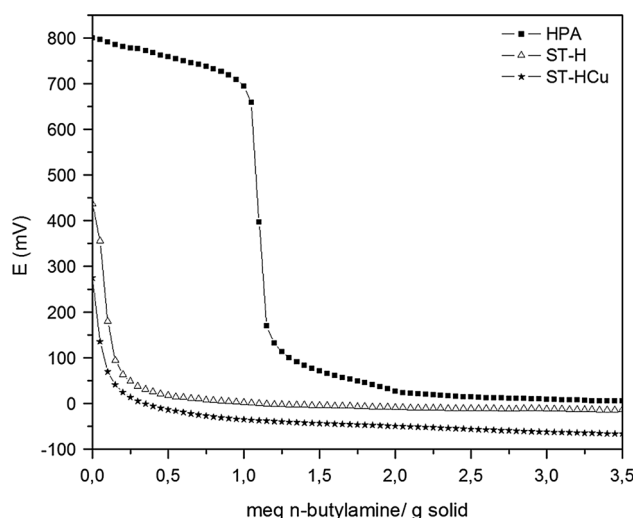
3.1.1 Potentiometric titration and textural properties

The initial electrode potential measured (E_i), the specific surface area (S_{BET}), the pore volume and the mean pore size of the prepared solids are shown in Table 2.

It should be noted that the SM-HCl sample synthesized with MTMS as precursor and HCl as catalyst could not be characterized due to its different gelification point.

Table 2 Acidity and textural properties of the solids synthesized

Sample	E_i (mV)	S_{BET} (m ² /g)	Pore volume (cm ³ /g)	Pore size (Å)
ST-HAc	137	464.0	0.2	18.7
ST-HCl	520	490.6	0.7	55.2
ST-H	440	321.1	0.2	22.9
ST-HCu	275	527.3	0.2	18.6
SM-HAc	17	2.6	–	43.3
SM-H	585	4.4	–	22.4
SM-HCu	260	6.0	–	103.5
STM-HAc	31	679.2	0.3	19.9
STM-HCl	530	520.5	0.8	63.4
STM-H	310	440.8	0.2	19.6
STM-HCu	370	524.5	0.2	16.9

**Fig. 1** Potentiometric curves of the ST-H, ST-HCu samples, and heteropolyacid (HPA)

As for the estimation of the acidic properties, Fig. 1 shows the potentiometric curves of different samples prepared with TEOS and the curve corresponding to the commercial heteropolyacid (HPA). In relation to the potentiometric titration, it is based on the difference in the electrode potential, which is determined mainly by the acid environment around the membrane, therefore, the measured electrode potential is an indicator of the acidic properties of the dispersion of solid particles. The initial electrode potential (E_i) indicates the maximum acid strength of the sites, and the value of meq amine/g solid, where the plateau is reached, indicates the total number of acid sites [28]. On the other hand, the maximum acid strength of the surface sites may be classified according to the following scale: very strong sites, $E_i > 100$ mV; strong sites, $0 < E_i < 100$ mV; weak sites, $-100 < E_i < 0$ mV; and very weak sites, $E_i < -100$ mV [29]. When acetic or hydrochloric acids were used in the synthesis, the variation

of E_i was important and, particularly, the quantity of acidic remaining sites in the solid when HCl was used. Incorporating HPA as catalyst, the potentiometric curve obtained was clean, with an initial high acidic strength and few acidic sites, meanwhile, the addition of Cu to the synthesis, monoprotic acidic sites were maintained, but the initial acidity decreased by the substitution of HPA protons by copper. When MTMS was used as precursor, the curves obtained were different at the initial acidity and the number of acidic sites. Although the acidity was lower when TEOS was used, these values should be appropriate for the use of the synthesized solids as antimicrobial fillers.

The samples where the silica precursors were mixed showed the same tendency for maximum acid strength and acidic sites, raising or lowering in a similar manner. Figure 2 shows the E_i values of all the samples, and an oscillation between values near 0 up to near 600 mV is observed. The acidity obtained in all the cases is sufficient to be used as fillers in the case of coatings.

As it can be observed in Table 2, the samples with higher S_{BET} values are those synthesized using TEOS as precursor, ST-HAc, ST-HCl, ST-H simple samples or STM-HAc, STM-HCl, STM-H mixed samples. In the case where only MTMS was used as precursor, S_{BET} is very low (almost coincident with the error of the technique), but when Cu is incorporated in the synthesis, the area increases. About the pore volume, the solids with higher value were those synthesized with HCl as catalyst. The N_2 adsorption–desorption curves showed by the synthesized samples indicate that the variation in the pore size and the surface area are consistent with the results of the curve hysteresis. High-quality hysteresis was observed for the samples prepared with the use of TEOS as precursor and when TEOS is mixed with MTMS.

3.1.2 X-ray diffraction and scanning electron microscopy

The XRD diagrams (not shown) of the ST-HCl, ST-HCu, STM-HCl, STM-HAc and STM-H samples showed

Fig. 2 Initial electrode potential of the synthesized samples

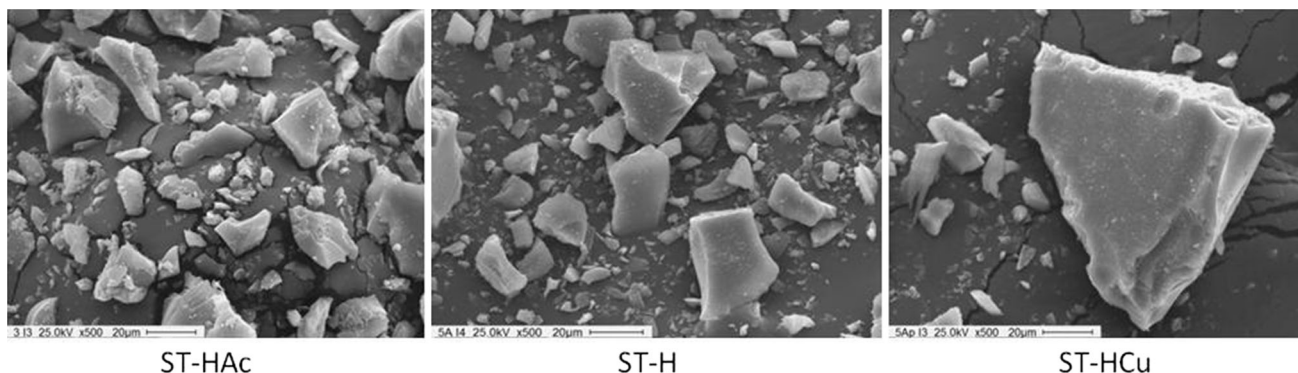
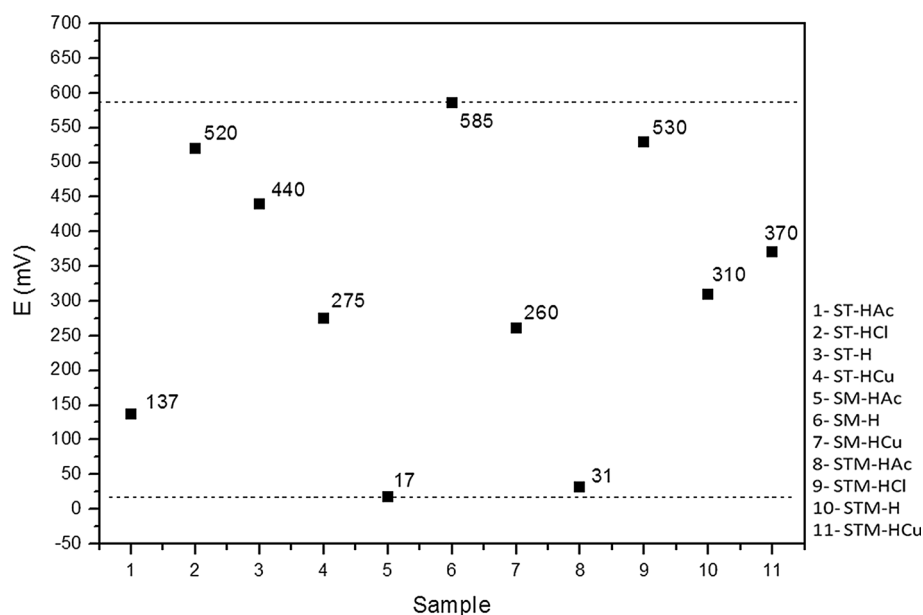


Fig. 3 SEM micrographs of the ST-HAc (pure silica), ST-H and ST-HCu samples

patterns with wide bands, assigned to amorphous silica, no matter which of the precursor was used.

On the other hand, the micrographs of the samples synthesized with TEOS, at a 500 \times magnification, are displayed in Fig. 3. They are similar in their morphology to the pure silica (ST-HAc sample) though in all the cases they are modified silica.

In relation with SEM of the SM-HCu sample (Fig. 4), a homogeneous distribution (mapping) of the components in the silica matrix was observed. The other samples containing Cu presented a similar behavior.

3.1.3 FT-IR

In order to understand the interactions that occur in the solids, the synthesized compounds were studied by FT-IR. When evaluating the effects of observable interactions in the FT-IR spectrum, it was decided to follow the method of identification through similarity of bands for the samples containing

HPA with Keggin structure. A broad band between 1100 and 964 cm^{-1} , and two bands at 800 and 470 cm^{-1} , which are the characteristic bands of silica, can be observed in all the samples. The HPA presents four characteristic bands at 1064, 962, 871 and 780 cm^{-1} . The spectrum of copper nitrate was also plotted in Figs. 5, 6 and 7 in order to compare with the spectra of the ST-HCu, SM-HCu and STM-HCu samples because Cu formed part of them in these solids. The HPA characteristic bands may be slightly shifted by interactions between the heteropolyacid and copper nitrate. In Figs. 5 and 6, the silica pure bands can be observed when TEOS or MTMS are used as precursors, and when mixing TEOS and MTMS to prepare the solids (Fig. 7), very similar bands to the previous cases were present.

Regarding Figs. 5 and 6, it is evident that the SM-HCu sample presented the bands of copper nitrate, but when TEOS was used in the ST-HCu sample, the main Cu bands disappear because copper strongly interacts with the HPA. But it is clear (Fig. 7) that when a mixture of precursors is

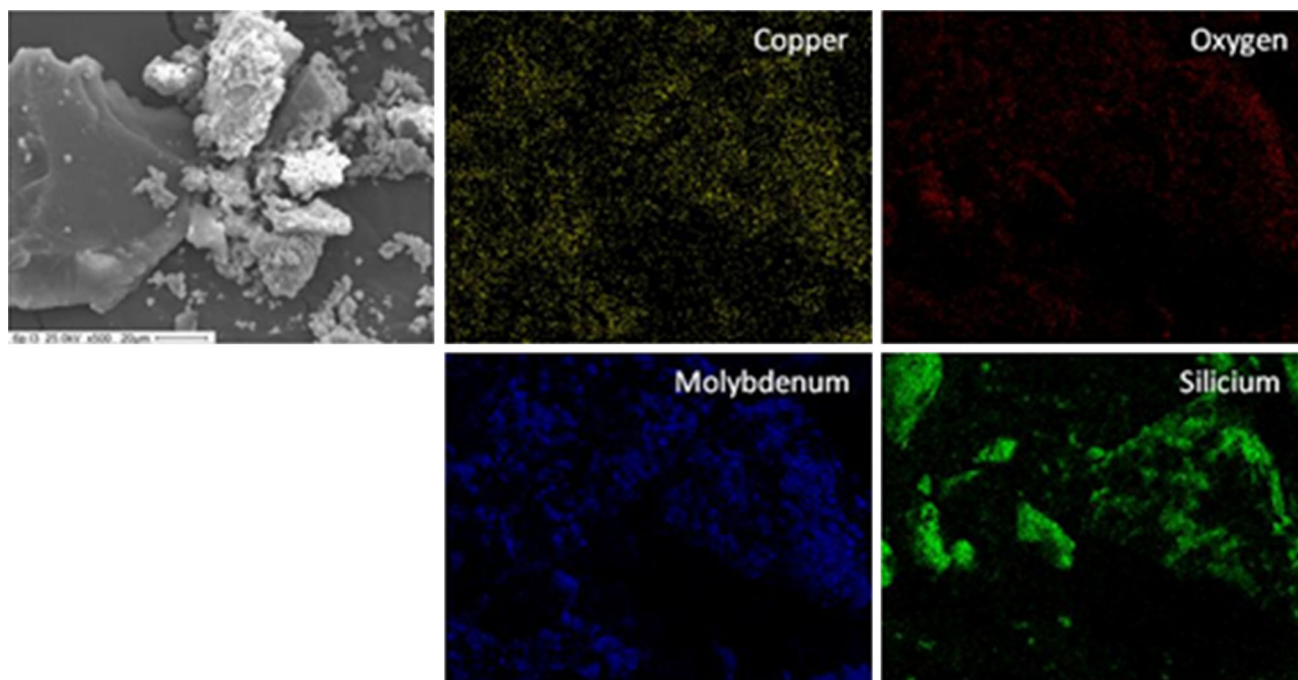


Fig. 4 SEM and mapping of the SM-HCu sample

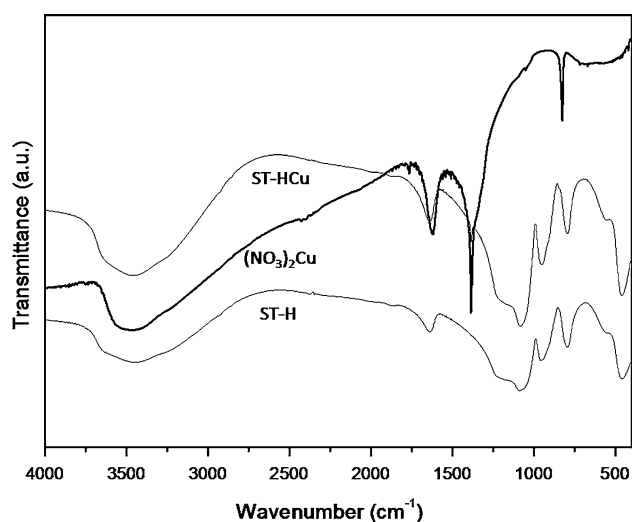


Fig. 5 FT-IR spectra of the synthesized ST-H and ST-HCu samples, and of bulk $(\text{NO}_3)_2\text{Cu}$

used, the Cu bands are faintly visible, which could be attributed to interaction between HPA and copper.

3.1.4 Antimicrobial activity

Antifungal activity was evaluated by measuring the fungal growth on CM-agar plates. The average diameter of each culture tested (*A. alternata* and *C. globosum*) for the different studied solids is shown in Figs. 8 and 9, respectively. Here it stands out the difference between fungal growth in

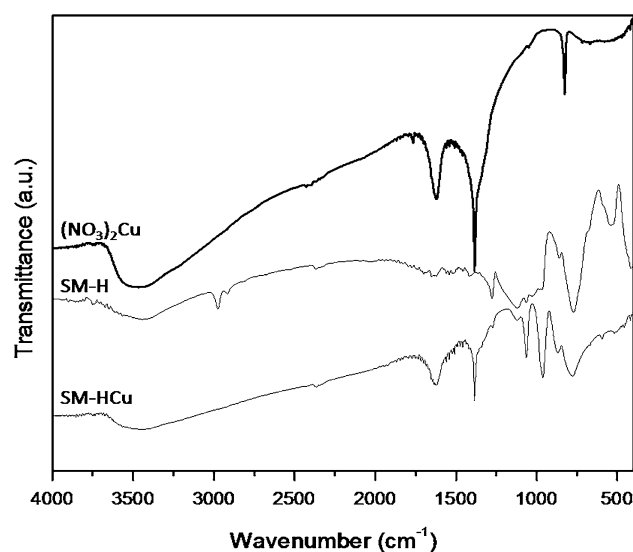


Fig. 6 FT-IR spectra of the synthesized SM-H and SM-HCu samples, and of bulk $(\text{NO}_3)_2\text{Cu}$

SM-HCu and STM-HCu samples and their respective solid controls; it seems that the addition of Cu provided greater inhibitory activity to the SM-H solid. Photographs taken with both isolates after an incubation period of 10 days are shown in Fig. 10. On the other hand, the solid ST-H showed a similar inhibitory effect regardless of the presence of Cu with both fungal isolates.

Table 3 shows the inhibition effect (%) against both fungal isolates determined according to Eq. (1). The SM-

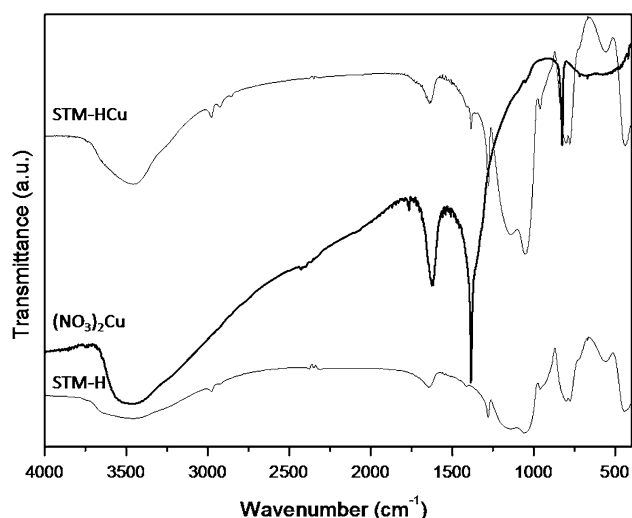


Fig. 7 FT-IR spectra of the synthesized STM-H and STM-HCu samples, and of bulk $(\text{NO}_3)_2\text{Cu}$

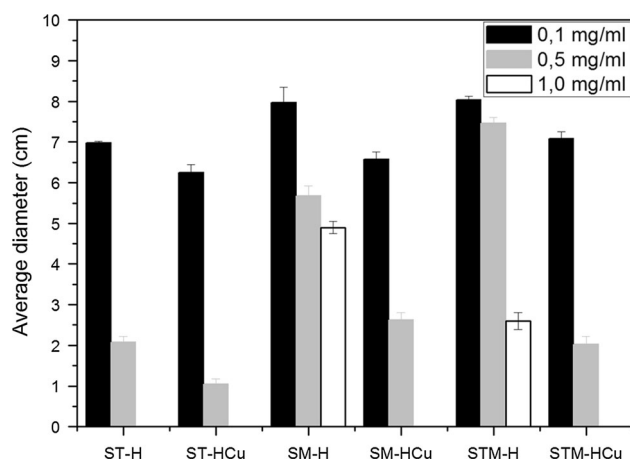


Fig. 8 *A. alternata*, average growth diameter with different solids containing Cu and their respective controls

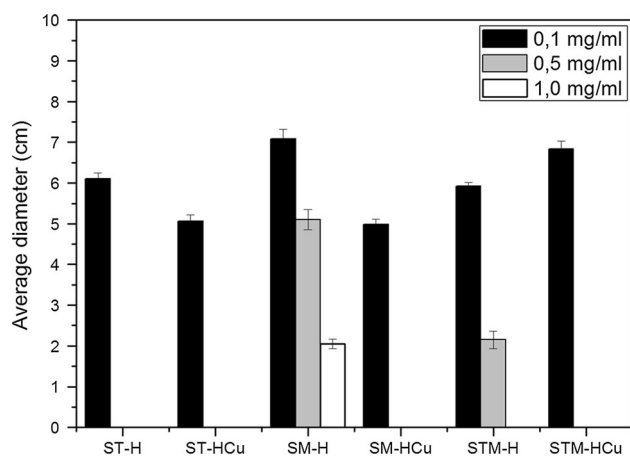


Fig. 9 *C. globosum*, average growth diameter with different solids containing Cu and their respective controls

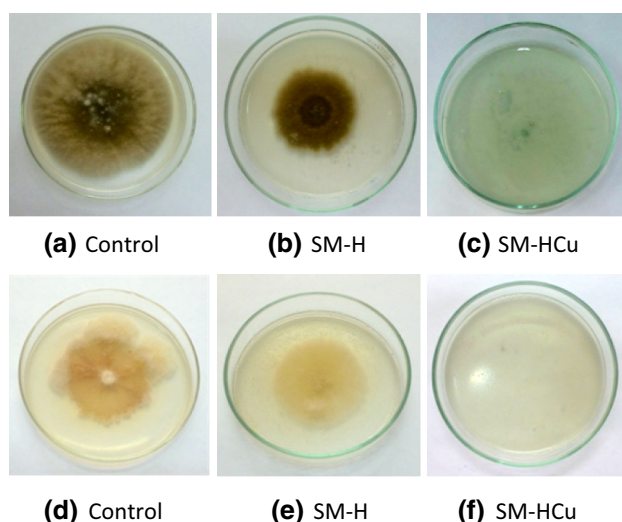


Fig. 10 *A. alternata* (a)–(c) and *C. globosum* (d)–(f) growth on CM-agar plates after 10 days at 25 °C, 1.0 mg/mL (b)–(c) and 0.5 mg/mL (e)–(f)

Table 3 Inhibitory activity of the modified silica matrix against *A. alternata* and *C. globosum* fungi for different copper content

Samples	Inhibition effect (%)					
	<i>A. alternata</i>			<i>C. globosum</i>		
	Copper concentration (mg/mL)					
	0.1	0.5	1.0	0.1	0.5	1.0
ST-H ^a	19.4	76.2	100	19.1	100	100
ST-HCu	24.0	78.8	100	31.7	100	100
SM-H ^a	8.1	34.6	43.4	6.0	32.4	72.8
SM-HCu	19.6	68.2	100	33.0	100	100
STM-H ^a	2.4	9.4	68.5	7.0	71.1	100
STM-HCu	14	75.3	100	9.3	100	100

^a Control

HCu and STM-HCu samples enhanced the antifungal activity compared to the copper free solids against both fungi. This effect is increased in the case of *C. globosum* fungus.

The MIC was defined as the lowest concentration of copper in the solids under study that produced a total inhibition of fungal growth. When tested, MIC was 1 mg/mL for *A. alternata* fungus and 0.5 mg/mL for *C. globosum* fungus. The results showed that the functionalized solids presented a higher inhibitory effect against *C. globosum* fungus.

4 Conclusions

The synthesis of the proposed solids to be used as biocides in antimicrobial coatings was possible to be carried out through the addition of copper. The evaluation of the

textural properties, acidity, morphology and antifungal activity was performed. In relation to the textural properties, it was observed an increase in the specific surface area (S_{BET}) when TEOS was used as precursor in the synthesis, instead of MTMS. When both precursors were used in order to obtain a mixed solid, the area was higher, increasing significantly with the addition of copper. Regarding the pore volume, the highest value is presented by those samples synthesized with HCl as a hydrolysis catalyst.

Concerning the acidity, when acetic acid or hydrochloric acid was used, the variation of the initial potential is evident, and the amount of remaining acid sites in the solid by the use of HCl is higher. When the HPA is used alone, the titration curve is clean showing high acidity though the acid sites are fewer; when copper is added to the synthesis, the shape of the potentiometric curve remains the same, resulting in monoprotic acid sites; however, when HPA protons are replaced with copper, the curve decreases. When MTMS is used as a precursor, the potentiometric titration curves appear different in initial acid strength and number of acid sites. However, even if the acidity is lower than when TEOS is used, it should be feasible for the synthesized solids to be used as antimicrobial fillers. In the case of the samples of silica prepared using a precursor mixture, with the HPA and copper, they all have the same trend of the acid sites and acid strength, moving up or down, even the behavior is similar to that when the precursor was used alone.

Though modified, the morphology of silica remains the same. By analyzing X-ray diffraction results, the amorphous characteristics of the obtained silica are evidenced and, regarding what was observed by FT-IR, the samples showed bands corresponding to the compounds with which silica was modified.

It was possible to evaluate the antifungal activity of the copper-doped solid through the inhibition test against the two fungi of interest (*A. alternata* and *C. globosum*) and the inhibition percentage determined for each case. SM-HCu and STM-HCu samples showed an increased antifungal activity by totally inhibiting the growth of both fungi, with lower concentrations compared to the other. Therefore, the methodology achieved in this preliminary study is successful to guide the selection of a suitable ecological biocide from a list of possible antifungal agents.

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