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Biological nanosilver particles for the protection of archaeological stones against microbial colonization

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abstract

The inhabitation of microorganisms and their subsequent interaction with mineral matrix of the stone substrate under varied environmental conditions encourages deterioration of stones leading to the loss of strength, durability and aesthetic. This study highlighted the synthesis of nanosilver particles (AgNPs) using the biogenic volatiles of the bacterial strain *Nesterenkonia halobia*. The antimicrobial activities of AgNPs were evaluated against the gram positive bacterial strain *Streptomyces parvulus* and fungal strain *Aspergillus niger*. Furthermore, the silver particles were mixed with two types of consolidation polymers and were used to coat the external surfaces of sandstone and limestone blocks. The stones treated with silicon polymer loaded with AgNPs showed an elevated antimicrobial potentiality against *A. niger* and *S. parvulus*. Scan electron microscope (SEM) and electron dispersive X-ray spectroscopy (EDX) analysis of treated stones demonstrated the existence of nano-composite structures containing the elemental silver. Polymers functionalized with AgNPs can be used not only as potent biocides but also for the consolidation of the historic monuments and artifacts.

1. Introduction

Sandstone and limestone are widely used in archaeological building in Egypt, for example sandstone was used in buildings of Egyptian temples. The temple of Edfu is one of these temples. It is located on the west bank of the Nile in the city of Edfu which was known in Greco-Roman times as Apollonopolis Magna, after the chief god Horus-Apollo. At the same time, there are many limestone archaeological buildings in Egypt such as the tomb of Teti's son Teti-ankh-km at Sakkara area. The temple of Edfu and the tomb of Teti's son Teti-ankh-km are exposed to various environmental and biological factors that result in apparent deteriorations of these monuments (Fig. 1). Heavy microbial colonization can obscure surface carvings, create an impression of poor maintenance and in some cases may promote the decay of the artifacts (Scheerer et al., 2009; Sterflinger, 2013).

Although, stones are extreme environments characterized with insufficiency of nutrients, enormous changes of humidity and elevated doses of UV radiation, they can be inhabited by various types of microorganisms causing stones deterioration (Selbmann et al., 2005). Deterioration mechanisms include acidolysis, accumulation of organic nutrients, discoloration, changes in the porosity, vapor diffusion changes and mobilization of ions (Gaylarde et al., 2003; McNamara and Mitchell, 2005).

Physical and chemical characteristics of the stone substratum in addition to the environmental conditions exert a direct impact on the selection of the colonization organisms. Cyanobacteria and algae comprehensively participate in stone biodeterioration via penetration and development of cryptoendolithic communities (Macedo et al., 2009; Hallmann et al., 2013). At the same time, fungi play a crucial effect in the disintegration of stones and archaeological materials due to their enormous enzymatic activity (Scheerer et al., 2009). Due to the high melanization of the fungal cell wall, stones colonized by fungi appear spotty or are even completely sheltered by black films. Moreover, fungi can not be easily killed by biocides or other antimicrobial treatments due to their thick cell walls (Sterflinger, 2010). In the meantime, bacteria are extensively involved in the deterioration of the historic stone structures (Warscheid and Braams, 2000). Different gram negative and gram positive bacterial strains such as *Cellulosimicrobium, Stenotrophomonas, Ochrobactrum, Lysobacter, Bacillus, Isoptericola*

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Fig. 1. Photographs clarifying the environmental and biological damage of the temple of Edfu in Upper Egypt (a, b, c) and the tomb of Teti's son Teti-ankh-km at Sakkara (d, e, f).

have been isolated from different artifacts (McNamara and Mitchell, 2005; Scheerer et al., 2009; Alisi, 2011). The potential degrading activity of bacteria takes place through the production of acids and surfactants (Stassi et al., 1998). Gram positive bacteria are more resistant to severe environmental conditions due to their capability to produce spores (Fajardo-Cavazos and Nicholson, 2006).

The removal of the microbial growths from stone surface is a complicated process because the conditions of the artifacts as well as the physical and chemical damaging factors must be considered very well. Actions against microbial growth can be mechanical, physical and/or chemical removal biodeteriogens. Many organic and inorganic compounds have been used as biocide agents to eliminate the biodeteriogens from cultural objects (Tiano, 1998).

Silver ions are known with their potent toxicity against broad range of bacteria, fungi, and viruses (Morones et al., 2005; Kim et al., 2007). Moreover, silver-based antimicrobials demonstrated positive properties such as thermal and chemical stability, environmental safety and low toxicity to human cells. These characters make silver-based materials suitable for wide varieties of applications (Lok et al., 2007; Pal et al., 2007).

Nanoparticles are of great interest due to their multiple potential applications (Knetsch and Koole, 2011). The nanometal particles have unique physicochemical properties including ultra small size, large surface to mass ratio, a distinctive reactivity with biological systems (Falletta et al., 2008; Zhang et al., 2011).

Various microorganisms including bacteria and fungi have been reported to produce metabolites that could be used for the synthesis of nanoparticles of different chemical compositions (Du et al., 2007; Verma et al., 2010; Ingle et al., 2011). The biologically synthesized nanoparticles are eco-friendly, cheap and completely safe. The aim of the current study was to evaluate the application of acrylic and silicon polymers loaded with biologically prepared nanosilver structures for the protection of two types of archaeological stones against bacterial and fungal colonization.

2. Materials and methods

2.1. Chemicals

Two types of consolidation polymers were used in this study; Primal AC33 polymer (Dow Chemical Co., USA) and silicon polymer (Wacker BS 1001, Wacker Chemei AG, Germany). The first consists of a mixture of methylacrylate and ethylmethacrylate while the second comprises of 50% silane/siloxane emulsion. Both types of the polymers were diluted to 3% final concentration with water.

2.2. Studied samples and sites

The sandstone and limestone samples were carefully picked up from the fallen fragments of the temple of Edfu and the tomb of Teti's son Teti-ankh-km, respectively. The selected sites for this study are clear example for the environmental and biological negative effect on these monuments.

2.3. Characterization of sandstone and limestone samples

The physical and chemical properties of the tested stones were studied using different techniques. A thin-section examination of the stones was carried out using polarizing light microscopy (PLM) with a Nikon Eclipse (C POL 600) microscope, equipped with an automatic photographic system and a digital camera (Model Nikon Coolpix 950). The mineralogical study included the analysis of samples by X-ray diffraction (XRD) using a Philips PW5 1337 automatic X-ray powder diffractometer with CuKα radiation. Patterns were obtained by step scanning from 3 °C to 75 °C 2 θ with a count for 0.5 s per step, exploration speed of 7 °C min^{-1} and 40 kV and 40 mA in the X-ray tube. At the time, stone samples were studied using scanning electron microscopy (SEM) model JEOL-6400, in order to determine the nature of crystalline texture and microstructure of the stones.

2.4. Preparation of the nanosilver particles

A batch bioreactor was used to prepare the composite metals structures of silver (Essa et al., 2005). The bioreactor composed of two chambers; one was used for bacterial growth (1 L, maintained under aerobic conditions by pumping in filtered compressed air), and the other chamber (100 ml) was used for metal precipitation by passing the culture exit gases through metal solution via a 0.2 µm filter to prevent bacterial contamination. In the growth chamber, about 800 ml of nutrient broth was inoculated with 100 ml of the Gram positive bacteria *Nesterenkonia halobia* (formerly *Micrococcus halobius*) in the mid-exponential growth phase (6 h) and was incubated at 30 ºC for 24 h. The culture outlet gases released from the growth chamber were passed into metal precipitation chamber that contains 100 ml $AgNO_3$ solution (0.1 mg/ml) for 30 min. After treating the metal solutions with the biogenic volatiles, the solution was subjected for ultra-speed centrifugation at 100,000 rpm, for 30 min. The collected silver particles were suspended in 10 ml deionized distilled H_2O then they were centrifuged again at 100,000 rpm for 30 min. This step was repeated three times and the collected silver particles were dried at 30 ºC for 24 h. A stock solution of the silver particles was prepared by suspending 10 mg of dried silver particles in 10 ml deionized distilled H_2O.

2.5. Antimicrobial activity of the composite metals structures

The antimicrobial activity of composite silver structures was assayed against the gram positive bacterium *Streptomyces parvulus* and the fungal strain *Aspergillus niger.* These strains were provided by the City of Science & Technology, Cairo, Egypt. To prepare the spore suspension of *A. niger,* the fungal culture was grown on potato dextrose agar slants and incubated at 25 ± 2 ºC for 7 days. Three milliliters of sterile distilled water were added the fungal slant and the fungal spore concentration was determined by haemocytometer. A volume of 100 ml of Potato dextrose broth medium amended with different concentrations of composite silver particles (20, 40, 60, 80 and 100 µg/ml) was distributed into 250 ml Erlenmeyer conical flasks. Then the flasks were inoculated with the *A. niger* spore suspensions (10^6 cell/ml). The flasks were incubated at 25 ºC on a rotary shaker at 120 rpm for 5 days. After incubation, the contents of the flasks were aseptically passed through preweighed Whatman No.1 filter paper to separate mycelial mat from culture filtrates. The filter papers along with mycelial mat were dried at 70 ºC until constant weight and the fungal biomass was calculated (mg/l).

To monitor the antibacterial activity of the composite silver structures, one milliliter of a fresh culture of *S. parvulus* (10^8 cell/ml) was used to inoculate 10 ml of nutrient broth supplemented with nanosilver structures at the concentrations mentioned above. After incubation for 48 h at 30 ºC, the bacterial growth was monitered by measuring the optical density spectrophotometrically at 600 nm.

2.6. Treatment of stone blocks with silver structures based on polymers

The composite silver structures were mixed with silicon and acrylic polymers at the concentration 40 µg/ml. Twenty five milliliters of the loaded polymers were used to coat all the surfaces of the stone blocks and were left 7 days at room temperature for complete drying.

2.7. Antimicrobial activity of the treated stones

To assay the antifungal activities of stones treated with polymers functionalized with silver particles, one surface of the treated stones was exposed to melted PDA containing spore suspension (200 cell/ml) of *A. niger.* Then, the stones were kept in humid cabinet at 25 ºC for 20 days. The fungal growth was measured visually as high growth (+++), medium growth (++), low growth (+) or no growth (-) on surface of the treated stones.

To assay the antibacterial activity of the treated stones, one surface of the treated stones was immersed in *S. parvulus* culture (3 x 10^6 cell/ml) for 2 h then they were incubated at 30 ºC for 24 h. Treated stones were immersed in 10 ml saline solution (0.85% NaCl) for 1 h with shaking. One milliliter of the washing solution was diluted 10 and 100 times with saline solution and 0.1 ml of the diluted solutions was plated on NA. After incubation at 30 ºC for 24 h the bacterial colonies were counted. Untreated stone samples were used as reference. The experiments were repeated three times with three replicates for each treatment.

2.8. Scanning electron microscope and energy dispersive analysis of X-rays of the composite silver structures based on silicon and acrylic polymers

Surface examination of the coated stones was carried out using scanning electron microscope (JEOL JSM-5410, Japan). The energy of the acceleration beam employed was 20 KV. EDX system attached with a JEOL JSM-5410 scanning electron microscope was used for elemental analysis or chemical characterization of the film formed on carbon steel surface before and after applying the synthesized inhibitor (V).

3. Results

3.1. Characterization of limestone and sandstone samples

Physical and mechanical properties of the stone samples including density, porosity, water absorption, compressive strength and tensile strength are summarized in Table 1. The obtained results clarified low density, high porosity and elevated water absorption capacity in the two types of the tested stones. At the same time, data obtained by polarizing microscope showed that the limestone sample comprised of fine grained calcite crystals besides iron oxides, quartz, clay minerals and fossils (Fig. 2a) while the sandstone consisted of white grains of crystalline silica in addition to clay minerals such as kaolinite and feldspars (Fig. 2b). Furthermore, the SEM images of the limestone samples showed salts crystallization and a clear disintegration between mineral grains (Fig. 2c). At the same time, voids were observed in sandstone samples (Fig. 2d). Additionally, the XRD patterns of the sandstone and limestone samples clarified that sandstone is consisting mainly of quartz with traces of kaolinite and feldspar but limestone sample comprises of calcite associated with dolomite and quartz as impurities (Fig. 2e and f).

3.2. Antimicrobial activities of the composite silver structures

As a result of pumping the biogenic volatiles of the bacterial strain *N. halobia* in the silver nitrate solution for 30 min exposure time, a light brown colloidal solution of composite silver structures was obtained. Data in Table 2 demonstrated the antimicrobial potentiality of the biologically prepared silver particles against *S. parvulus* and *A. niger.* A clear suppression in the growth of the tested microorganisms was recorded with the different concentrations of the silver particles. The microbial growth was completely vanished at the concentration 60 µg/ml and above while at 40 µg/ml the percentage of growth reduction reached 92.5% with *S. parvulus* and 93.8% with *A. niger.*

Table 1
Physical and mechanical properties of limestone and sandstone samples.

Analysis	Limestone	Sandstone
Bulk Density (g/cm^3)	1.9	1.6
Water Absorption (%)	8.4	19.8
Porosity (%)	15.6	26.3
Compressive strength (MPa)	26.9	19.8
Tensile Strength (MPa)	4.3	3.2

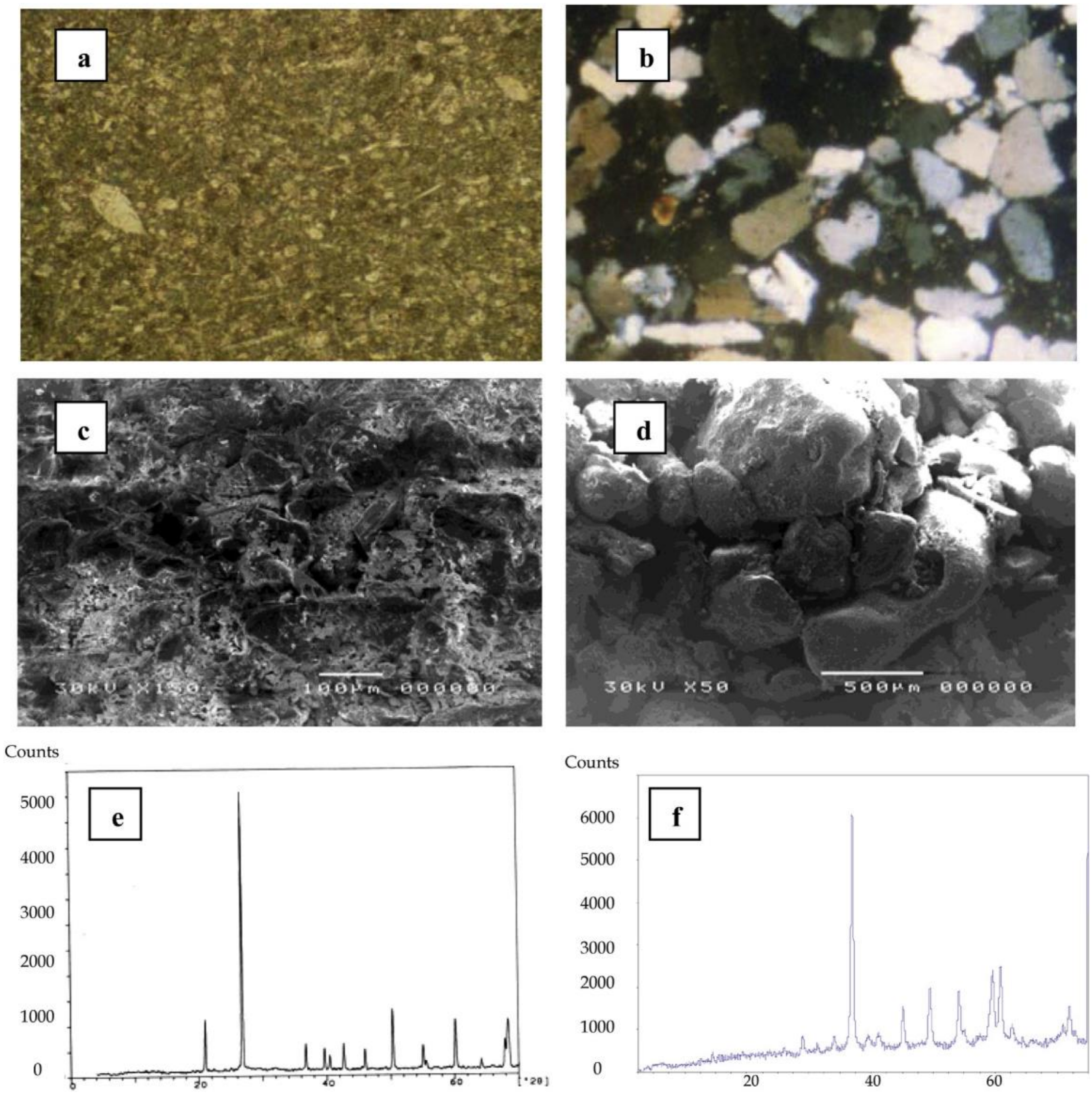

Fig. 2. Thin section of limestone (a), sandstone (b), SEM micrograph of limestone showing erosion and disintegration between mineral grains (c), sandstone showing presence of voids due to the dissolving and losing of binding materials (d), XRD pattern of sandstone (e) and XRD pattern of limestone (f).

3.3. The antimicrobial activity of the stones treated with composite silver structures based on polymers

Although the AgNPs at the concentration 60 µg/ml demonstrated a complete disappearance of the tested strains, the concentration 40 µg/ml was chosen to be mixed with the two types of the polymers in order to avoid any colorimetric changes in the treated stones. In this experiment, all the surfaces of the stone blocks were coated with the silicon and acrylic polymers functionalized with the silver structures. Data in Table 3 showed the antibacterial activities of the treated stones against *S. parvulus*. The treated sandstone blocks recorded a remarkable reduction in the percentage of the bacterial cell recovery 98.4% with the functionalized silicon polymer and 97.2% with the functionalized acrylic polymer. At the same time, the treated limestone blocks demonstrated clear suppression in the percentage of the *S. parvulus* cell recovery 98.6% with the functionalized silicon polymer and 97.1% with the impregnated acrylic polymer.

Regarding the antifungal activities of the treated stones, data in Fig. 3 demonstrated variable degrees of antifungal potentiality. A sharp growth inhibition of *A. niger* on the surface of the sandstone and limestone blocks coated with silicon or acrylic polymers loaded with composite silver structures comparing with the prominent growth of *A. niger* on the surfaces of the untreated stones.

Table 2
Antimicrobial activity of different concentrations of the nanosilver particles against the bacterial strain *Streptomyces parvulus* and the fungal strain *Aspergillus niger*. The bacterial growth was monitored as culture optical density (OD_{600}) while the fungal growth was assayed as biomass dry weight. Data are the means of three replication ± standard errors.

A. niger		*S. parvulus*	
AgNPs (µg/ml)	D.wt (mg/l)	AgNPs (µg/ml)	Optical density (D_{600})
0	186.1 ± 10.8	0	1.34 ± 0.19
20	154.0 ± 8.7	20	0.86 ± 0.25
40	11.6 ± 7.6	40	0.10 ± 0.24
60	0.0	60	0.0
80	0.0	80	0.0

Table 3
Streptomyces parvulus cell recovery from sandstone and limestone blocks that were treated with silicon (S) and acrylic (AC) polymers loaded with nanosilver particles (40 mg/ml).

Control	Limestone blocks	Sandstone blocks
S	S with AgNPs	
9.37 10^{5}	1.33 10^{4}	1.43 10^{4}
AC	AC with AgNPs	
9.78 10^{5}	2.86 10^{4}	2.67 10^{4}

3.4. SEM & EDX analysis of the composite silver structures based on polymers

Data in Fig. 4 showed the scanning electron microscope (SEM) analysis of the treated and untreated polymers with composite silver structures. In case of the functionalized polymers, tiny particles (10-20 nm in diameter) were identified in the treated silicon and acrylic polymers meanwhile these particles were completely absent in the unloaded polymers. At the same time, the EDX analysis of these minute structures showed the presence of the elemental silver in the treated silicon and acrylic polymers in addition to oxygen, sulfur, silicon, aluminium, calcium and carbon. Furthermore, the EDX analysis of the unfunctionalized polymers showed the presence of carbon, oxygen, chloride, calcium and silicon peaks in case of the acrylic polymer while the peaks of carbon, silicon and oxygen were recognized with the silicon polymer.

4. Discussion

The distribution of moisture within stone mainly depends on pore-size and pore distribution in addition to the environmental conditions such as temperature and humidity levels (Garland and Rogers, 1995). The physical and mechanical properties of limestone and sandstone samples clarified low density, high porosity and high water absorption. Moreover, the EDX and XRD analysis revealed the presence of calcite crystals besides iron oxides and clay minerals in the stone samples. The presence of such these salts affect directly on the absorption of moisture (Kozlowski et al., 1992). The absorbed water promotes the dissolving of these salts causing loss of the binding materials between stone grains which in turn induces the collapse of the internal structure of stone matrix. At the same time, the dissociation of these salts decreases the mechanical properties of stones such as compressive and tensile strength resulting in stone damage and degradation. As a result of the deterioration process, the disintegrated stones become appropriate substrate for microbial colonization (Espinos et al., 2010; Laho et al., 2010; Abd El-Rahim and Khallaf, 2011). The use of consolidation polymers is crucial to improve the physical and mechanical properties of the deteriorated stones and simultaneously to suppress the inhabitation of microorganisms on the surfaces of stone materials (Ross et al., 1990; De Leo et al., 2012).

Fig. 3. The antifungal activity of the acrylic polymer and silicon polymer loaded with nanosilver structures (40 mg/ml) against *Aspergillus flavus*: (A) represents limestone blocks treated with functionalized silicon polymer (2) and functionalized acrylic polymer (3) while (B) represents the sandstone blocks treated with functionalized silicon polymer (2) and functionalized acrylic polymer (3). Stone blocks number 1 in both (A) and (B) represent the untreated stones.

In a previous study (Essa et al., 2012), the bacterial strain *N. halobia* demonstrated a high capability for the precipitation of different metals via the volatile metabolites of the culture off-gases. In the present work, AgNPs were prepared biologically via exposing the Ag^{+} ions to the biogenic gases produced during the aerobic growth of *Nesterenkonia halobius*. Within thirty minutes, the soluble silver ions were transformed into colloidal silver solutions. Ammonia in the bacterial biogases that was confirmed using Nessler's solution is responsible for the transformation of different metal ions into nitrogen-based metal complexes. The release of ammonia during the bacterial growth was attributed to enzymatic processes of the organic matter decomposition (Kuok et al., 2013).

When silver nitrate was dissolved in water, hydrated cations were formed. These cations undertake some chemical reactions under alkaline conditions due to the existence of ammonia to attain more stable forms. At low concentration of ammonia, silver hydroxide ions are formed. The continuous increase of ammonia concentration in the precipitation chamber of the bioreactor will react with the silver hydroxide resulting in the formation of diammine-silver(I) complex (Essa et al., 2012).

The transformation of the soluble silver ions into colloidal silver particles mainly depends on the exposure time to the biogenic volatiles. At short exposure time, tiny silver particles with 10-20 nm in diameter were formed as showed in the SEM analysis. However, the presence of elemental sulfur in the composite silver structures could be attributed to the occurrence of volatile sulfur metabolites in the bacterial biogas. These compounds have a great tendency to chelate and precipitate different metals out of their solutions (Essa et al., 2005).

The antimicrobial activity of the AgNPs was evaluated against the Gram positive bacterial strain *S. parvulus* and the fungal strain *A. niger*. The obtained results clarified a potential antibacterial activity of AgNPs. The inhibitory action of silver on the growth of microorganisms could be attributed to the negative effect on DNA replication resulting in inactivated expression of vital cellular proteins (Yamanaka et al., 2005). It has also been hypothesized that silver ions can bind with sulfhydryl groups of proteins affecting the function of membrane bound enzymes of the respiratory chain (Lok et al., 2006; Rai et al., 2009). At the same time, the direct interaction of AgNPs with cell membranes affects ions transportation and cause membrane perforations (Sondi and Salopek-Sondi, 2004). Finally, the accumulation of excessive amounts of reactive oxygen species (ROS) as a result of the exposure to silver might induce cell death (Choi and Hu, 2008).

Microbial colonization on the surfaces of stone materials leads to negative impacts including physical and chemical deterioration of the stone. Biodeterioration process is the result of complex activities and interactions of the microbial communities with the stone matrix. Different inorganic materials such as titanium dioxide and Ag-doped titanium dioxide have been tested with consolidants and applied on stone surfaces against biological colonization (La Russa et al., 2012; Ruffolo et al., 2013). In the present investigation, the silicon and acrylic consolidant polymers that were functionalized with AgNPs demonstrated affirmative consequence on

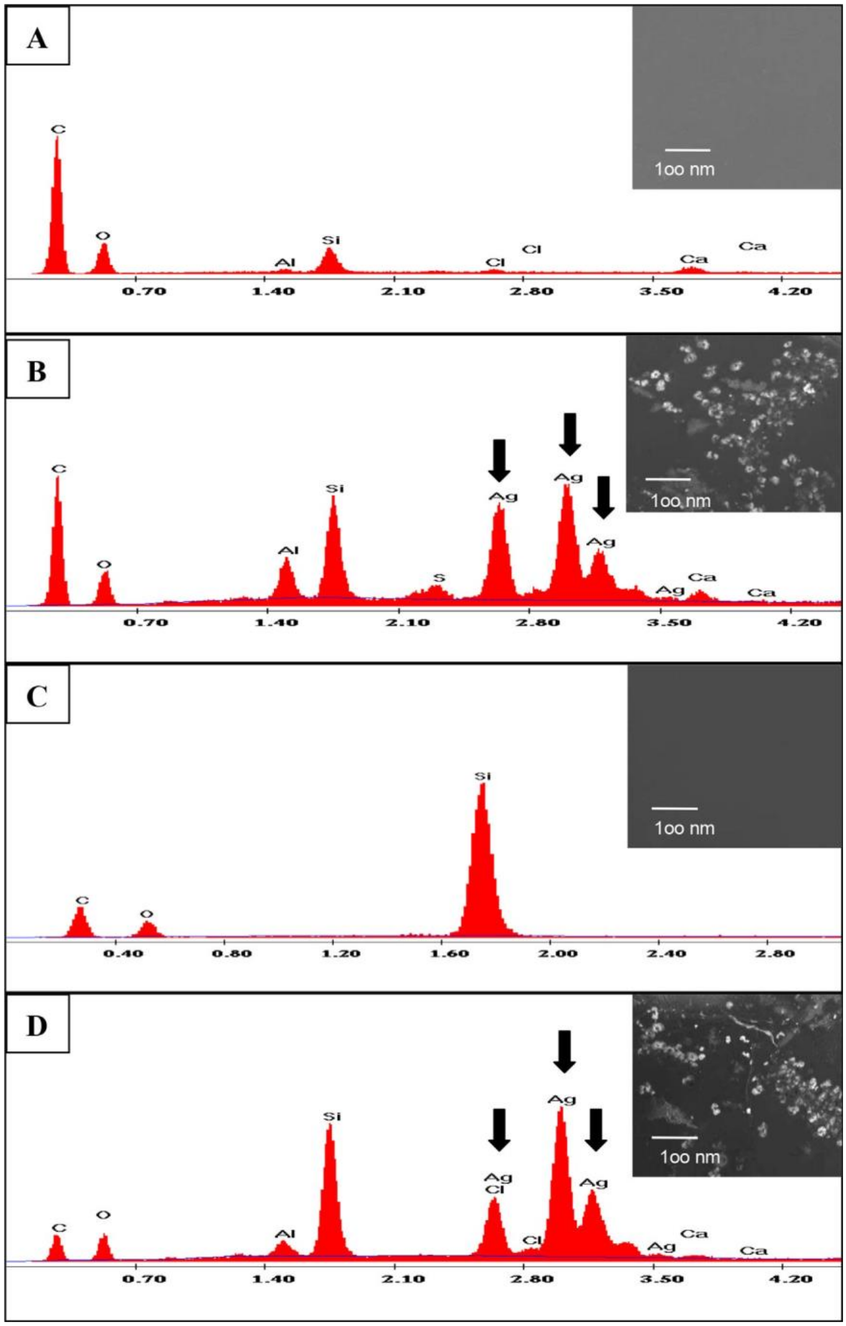

Fig. 4. SEM images and EDX analysis of the unfunctionalized silicon polymer (A), functionalized silicon polymer (B) and unfunctionalized acrylic polymer (C), functionalized acrylic polymer (D) while black arrows indicate the elemental silver.

the treated stones via the suppression or prevention of the growth of *S. parvulus* and *A. niger.* In agreement with these findings, Pinna et al. (2012) demonstrated an efficient preservative treatment against the microbial growth on stone materials treated with copper nanoparticles mixed with consolidants and water-repellents. This treatment resulted in positive effects for the protection of stones from biodeterioration.

This study clarified a remarkable antibacterial and antifungal potentiality of the biologically prepared AgNPs and at the same time their activity does not altered upon combining with silicon and acrylic consolidation polymers. These results are in agreement with Bellissima et al. (2013) who investigated the effectiveness of stone samples covered with a grafting agent mixed with chemically prepared silver nanoparticles for the suppression of bacterial colonization. They recorded a marked reduction of the cell viability of Bacillus subtilis on the surface of the treated stones.

5. Conclusion

In this study AgNPs was synthesized biologically via volatile metabolites produced during the aerobic growth of *N. halobius*. The prepared AgNPs demonstrated high antimicrobial activity against *S. parvulus* and *A. niger.* AgNPs was applied to the surfaces of sandstone and limestone blocks via incorporating them with consolidation polymers. The stones treated with functionalized polymers demonstrated a sharp reduction in growth of *S. parvulus* and *A. niger*. Moreover, SEM and EDX analysis showed the presence of nanosilver particles on the surface of the treated stones. The consolidation polymers loaded with AgNPs presents a potential application not only for the solidification of old stones but also for the protection against bacterial and fungal colonization. Further studies are required to assess the application of the consolidation polymers integrated with nanosilver particles in situ treatment.

Conflict of interest

The authors declare that there is no conflict of interest.

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