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(Research Paper)

Enhancing the Antimicrobial Efficacy of Acrylic Polymers through *in situ* Biological Synthesis of Copper Nanocomposites Used in Restoration of Stony Cultural Heritage

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Abstract:

Acrylic polymer is widely used as a protective material for coatings, bridges, ships and locomotives. Additionally, these synthetic polymers are used in the treatment of stone buildings, acting as bonding agents and protective layers. The synthetic polymers can be subjected to various types of degradation, including chemical, physical and biological deterioration. Microorganisms as biological agents can also damage the structure and functionality of synthetic polymers. To enhance the structural integrity and functionality of polymers, nanoparticles can be incorporated into polymers to improve their physical properties and antimicrobial capabilities. Plant extracts can be utilized to synthesize metal nanoparticles. The biological synthesis is a simple, cost-effective and environmentally friendly method.

In this study, an *in situ* method was used to produce the nanocomposite. First, precursors such as aqueous extract of *Juglans regia* and acrylic polymer were mixed together. This reaction resulted in the formation of nanoparticles within the polymeric structure. The morphological characteristics of the nanocomposite were then studied using Field Emission Scanning Electron Microscopy (FESEM). The presence of copper in this nanocomposite was further analyzed using Energy-Dispersive X-ray Spectroscopy (EDX), where the CuKα and CuKβ peaks confirmed the presence of copper atoms. The antimicrobial activity of the nanocomposite was tested against several strains of bacteria and fungi, including *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Cladosporium cladosporioides*, *Aspergillus niger* and *Alternaria alternata*. The antimicrobial activity of the nanocomposite was compared with the acrylic polymer. The nanocomposite synthesized by the *in situ* method demonstrated higher antimicrobial activity than the acrylic polymer. While the polymer alone could only reduce the number of bacterial and fungal cells by one logarithm, the nanocomposite achieved a reduction of 2-4 logarithms. With these improved antimicrobial properties, it can be concluded that the bioengineered nanocomposite has reasonable potential for use as an antimicrobial coating of stone surfaces.

Keywords: Antimicrobial coating, Biofilm, Biosynthesized metal nanoparticles, *In situ* synthesis, *Juglans regia*, Nanocomposite, Acrylic polymer

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Introduction

The production of polymers dates back to the 19th century. Acrylic polymers are widely used in various fields due to their attractive appearance and smooth, high quality, wash ability, and resistance to dirt and scrubbing. Applications include latex paints, architectural coatings, medical devices, the manufacture of trains and boats, channels, military weapons, adhesives and sealants (1).

Acrylic polymers are primarily composed of acrylate or methacrylate monomers, which are highly stable against light, heat and chemicals. These coatings are cost-effective to produce and are resistant to weathering, pollution, acids and alkalis. Their exceptional weather resistance makes them ideal for outdoor use (2). A notable feature of exterior coatings is their ability to prevent the aging of architectural and historical monuments (3).

In the 1960s, synthetic polymers were considered as a means of slowing down the biodeterioration of cultural heritage. Despite their frequent use in the conservation of cultural heritage, their resistance to microbial colonization was not initially measured (4). However, Cappitelli *et al*. demonstrated that fungi could not only attack acrylic treated marble, but also thrive on these treated stones (5). Additionally, bacteria can colonize stone surfaces, with Gram-positive chemoorganotrophic bacteria, particularly *Bacillus* spp., and closely related genera, commonly isolated from edifices. Bacillus species can survive harsh environmental conditions due to their ability to produce spores (6) and have the potential to damage stone surfaces through the production of acids and surfactants (7).

Microbial degradation of polymers can occur in several ways: (i) alteration of surface properties, (ii) increased nutrient availability due to degradation of monomers and additives, (iii) production of metabolites such as acids and enzymes, (iv) microbial infiltration and physical disturbance of structures, (v) water accumulation, and (vi) excretion of pigments (8).

Acrylic polymers have been shown to have antimicrobial properties against bacteria and fungi (9). Therefore, enhancing these antimicrobial properties can increase resistance to biodeterioration and prevent the colonization of harmful microorganisms. The performance of polymers can be further enhanced by incorporating nanoparticles as additives into acrylic polymers (10, 11). Today, nanotechnology enables the design of nanocomposites that are highly compatible with the original substrate, allowing nanomaterials to penetrate deeply into weakened substrate materials due to their small particle size (12, 13). Recent advances in nanoparticle synthesis have significantly improved the ability to control the size, composition and uniformity of nanoparticles (14). Biological methods for nanoparticle production offer several advantages, including high efficiency, cost-effectiveness, use of waste materials such as fruit and vegetable peels, and environmental friendliness (15, 16). Plant based aqueous extraction for nanoparticle synthesis offers advantages over traditional methods, such as safer operation, the ability to stop processes, and the production of diverse products (17-19).

Copper nanoparticles in particular have demonstrated multi-toxicity against a wide range of bacterial species. They have attracted attention for their antimicrobial activity at lower cost and greater availability, compared to metals such as gold and silver. Copper nanoparticles can produce oxidative stress and alternate between two oxidation states, cupric (Cu II) and cuprous (Cu I), which distinguishes them from other metal nanoparticles. Finding a simple method to incorporate copper into polymers to improve their stability remains a challenge. Both *in situ* and *ex situ* methods have been previously used to prepare polymer/TiO_2 nanocomposites (20, 21). In *ex situ* synthesis, nanoparticles are prepared separately and then added to the polymer in a two-step process. In contrast, *in situ* synthesis involves a one-step production of nanocomposites using the corresponding precursors, resulting in a homogeneous dispersion of nanoparticles within the polymer matrix (2, 21).

This study focuses on the one-step biological production of copper nanocomposites using an *in situ* method. The use of this bioengineered polymer offers a promising solution for the consolidation of stone materials.

Materials and methods

Microorganisms

Bacillus subtilis ATCC 6633, *Pseudomonas aeruginosa* ATCC 15442, *Cladosporium cladosporioides* ATCC 16022, *Aspergillus niger* ATCC 16404, *Alternaria alternate* ATCC 34957 were used in this study. Such microbial species have been repeatedly isolated from various ancient building and stone materials, and therefore were used as microorganism models to evaluate nanocomposites with antimicrobial properties. As can be seen, due to the high diversity of deteriogenic microorganisms on the surfaces of historical monuments, both bacteria and fungi were used for evaluation. These isolates are kept in the microorganism bank of the Department of Microbiology, Alzahra University. The bacterial isolates were cultured on Tryptic Soy Agar (TSA), and stored at 4 °C. The fungal strains were cultured on Potato Dextrose Agar (PDA), and stored at 4 °C.

Biosynthesis of nanocomposites by *in situ* method

The green husk of *J. regia* was obtained from Karaj Gardens (Karaj, Alborz province, Iran) and washed with soap and tap water. It was then air dried, and 1.6 g of its dried powder was added to 58 ml of boiling distilled water. The suspension was then placed in a water bath at 100 °C for 10 min and filtered through Whatman paper no. 1. Then, 150 µl of *J. regia* extract and 10 ml of 1 mM copper acetate (Merck) were added to 2% w/v acrylic (22). The mixture was kept at room temperature in the dark. The formation of nanocomposites was followed by the color changes (23, 24). Then, the nanocomposites were sectioned for electron microscopy images.

Preparation of the experimental model

Limestone was prepared with surface dimensions of 1.5 x 1.5 x 1.5 cm. After polishing the surface, it was washed with distilled water, dried at 105 °C for 24 h, and then cooled at room temperature. Finally, 50 µl of the nanocomposites were applied to the stones with a brush. This step was repeated three times with an interval of 2 h (25). Water absorption, density and porosity of the nanocomposites were determined (26).

The absorbed water was calculated by following formula (10, 27):

$$\text{Water absorption} = \frac{W_2 - W_1}{W_1} \times 100$$

$$\text{Density} = \frac{W}{V}$$

$$\text{Porosity} = \frac{W_2 - W_1}{V} \times 100$$

W1: Stone mass before immersion
W2: Stone mass after 24 h of immersion
W: Stone weight
V: Stone volume

Characterization of nanocomposites using FESEM and EDX techniques

The EDX method was performed using a Scanning Electron Microscope (FESEM MIRA3/EDX) (TESCAN/ Czech Republic) to validate the presence of copper atoms on the polymer. The FESEM method was carried out using the FESEM MIRA3 TESCAN and the particle size and its distribution were calculated using the image tool UTHSCSA, version 3.00.

Determination of the antibacterial activity

The Kirby Bauer disk diffusion technique with slight modifications was used to determine the bactericidal impact of this composite. For this purpose, 3 × 3 cm square pieces of aluminium foil were coated with 150 µl of nanocomposite and acrylic (control) using brush. This was repeated three times with an interval of 2 h between each brushing. *Bacillus* sp. is one of the most common bacteria isolated from monuments, with chemoorganotrophic and endospore forming properties and resistant to environmental stress was chosen as a model for this test (28). Due to endospore formation, this bacterium shows high resistance to many antimicrobial agents.

Concentrations of 5×10^7 CFU/ml were prepared from fresh bacterial cultures. The bacterial concentration was measured using a spectrophotometer (HACH LANGE) at a wavelength of 600 nm (OD600). Then, 100 µl of each bacterial suspension was cultured on Luria-Bertani (LB) agar containing 1% agar. These coated aluminium foils were placed on the inoculated LB agar and incubated at 30 °C for 24 h. The aluminium foils were then removed and the plates incubated again overnight. The grown bacterial colonies were scored and photographed (29).

Determination of antimicrobial activity of nanocomposite and acrylic polymer

The agar slurry was prepared by adding 0.85 g NaCl and 0.3 g agar-agar to 100 ml of deionized water. The medium was then sterilized by autoclaving, and cooled to 45 °C. Next, 3.0 × 3.0 cm square aluminium foils containing nanocomposite and acrylic polymer were prepared. Each foil was placed in a sterilized plate. Then, it was mixed 100 ml of agar slurry at 45 °C with 1.0 ml of microbial suspension. The final microbial concentration in the molten agar slurry should be 10^6 cells/ml for bacteria and 10^5 cells/ ml for fungal spores. One ml of this mixed slurry was then pipetted onto the nanocomposite and acrylic polymer to form a 1 mm thick film. After solidification of the agar slurry, the plates were incubated in the incubator (Memmert, Germany) for 24 h at 37 °C for bacteria, and 96 h at 25 °C for fungi. Serial dilutions of the agar slurry were prepared from time point "0" h. To determine the recoverable CFU, each dilution was plated on TSA and SDA for bacteria and fungi, respectively. The plates were then incubated and colonies from each dilution were counted to calculate log reduction as described by Agents and Sawant (2011).

Determination of antimicrobial activity of treated stone

Stone surfaces (1.5 × 1.5 cm^2) were treated with 50 µl of nanocomposite and acrylic polymer (control). After drying, the stone samples were placed on sterilized plates. Then, 50 µl of *B. subtilis* at a concentration of 10^5 CFU/ml was added to the surface of the treated stone samples. After 30 min, 10 ml of sterilized saline was added and the plates were shaken for 30 min. Dilutions of 1:10 and 1:100 were then prepared. To count bacteria, each bacterial dilution was cultured on TSA and incubated at 37 °C for 24 h. The number of colonies grown from each dilution was counted. Each assay was performed with a minimum of three replicates. The test steps are shown schematically in Fig. 1. The reduction in bacterial viability was investigated by comparing the final microbial count (FMC) of stones treated with nanocomposite and acrylic polymer (25).

Percentage of antimicrobial effect= FMC (acrylic polymer) - FMC (nanocomposite)/FMC (acrylic polymer) × 100

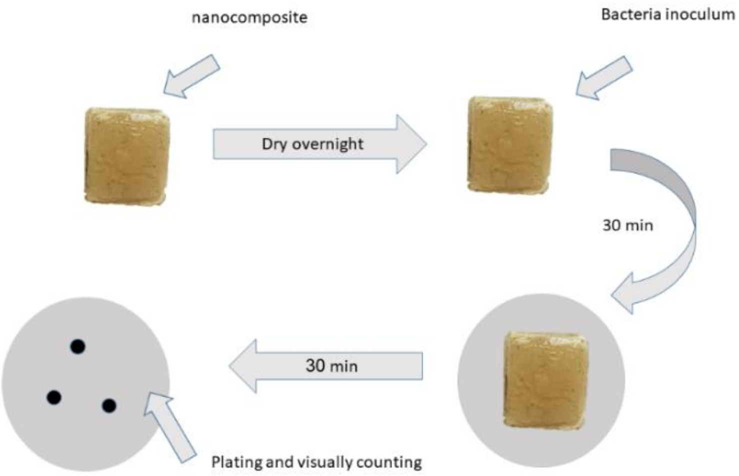


Fig. 1. Determination of antimicrobial activity of nanocomposite and acrylic polymer by deposition and recovery method

Biofilm formation on nanocomposite

The surfaces of glasses (~1 × 1 cm) were gradually covered with 150 µl of nanocomposite, and this was repeated 3 times. Then, 1 × 10^7 CFU/ml of *P. aeruginosa* was prepared in LB broth. Next, 5 ml of this bacterial suspension was added to the sterile tube. The coated glass surfaces were placed in these tubes to form a biofilm. The tubes were then incubated in a shaker-incubator at 37 °C and 250 rpm. The glass pieces were taken out after one, two, and three days. They were gently washed three times with 0.1 M phosphate buffer (pH 7.4), and placed in fixation buffer (1% v/v glutaraldehyde and 4% v/v formaldehyde) overnight. Then, the glass pieces were re-rinsed 3 times with the same phosphate buffer and dehydrated in serial dilutions of acetone (25%, 50%, 75%, 90% for 5 min, and 100% for 5 min 2 times). Finally, the glass pieces were dried with CO_2 at critical point (BAL-TEC CPD 030 Critical Point Drier), coated with gold and finally observed by FESEM (29).

Results

In situ biosynthesis of the nanocomposites

For the *in situ* biosynthesis of the nanocomposite, the color change of the nanocomposite was checked weekly. After confirming the stability of the color of copper nanocomposites, the sectioned nanocomposite was observed by electron microscopy (Fig. 2). As shown in Fig. 2A and 2B, the FESEM micrographs clearly reveal the presence of spherical nanoparticles deposited directly adjacent to the polymer. The FESEM image in Fig. 2B shows the spherical shape of the nanoparticles. As indicated in Fig 2B, this nanocomposite was synthesized with dimensions ranging from 22 to 44 nm and was uniformly embedded on the polymer.

The nanocomposite was analyzed using EDX. The CuKα and CuKβ peaks in Fig. 3A confirm the presence of copper atoms, while Fig. 3B verifies the dispersion of copper atoms throughout the polymer.

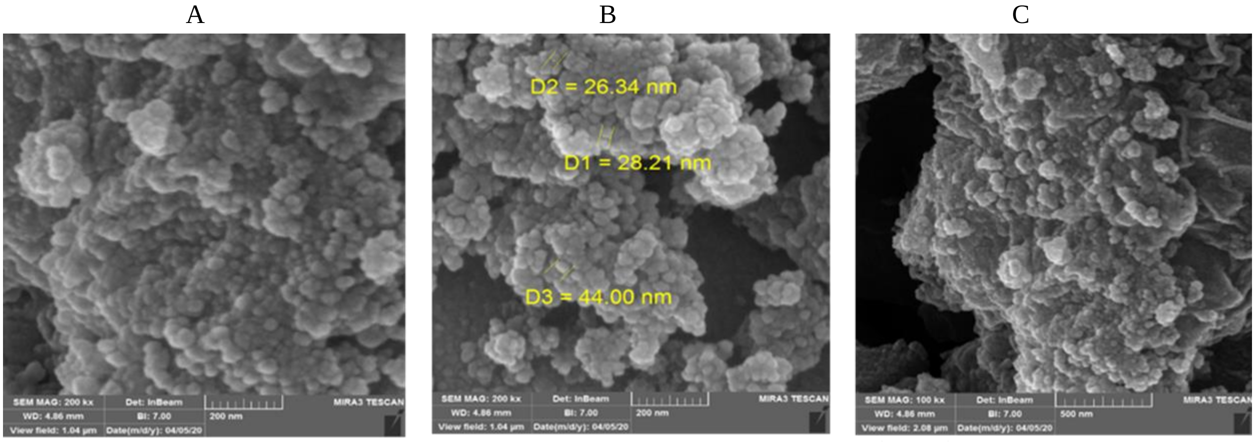
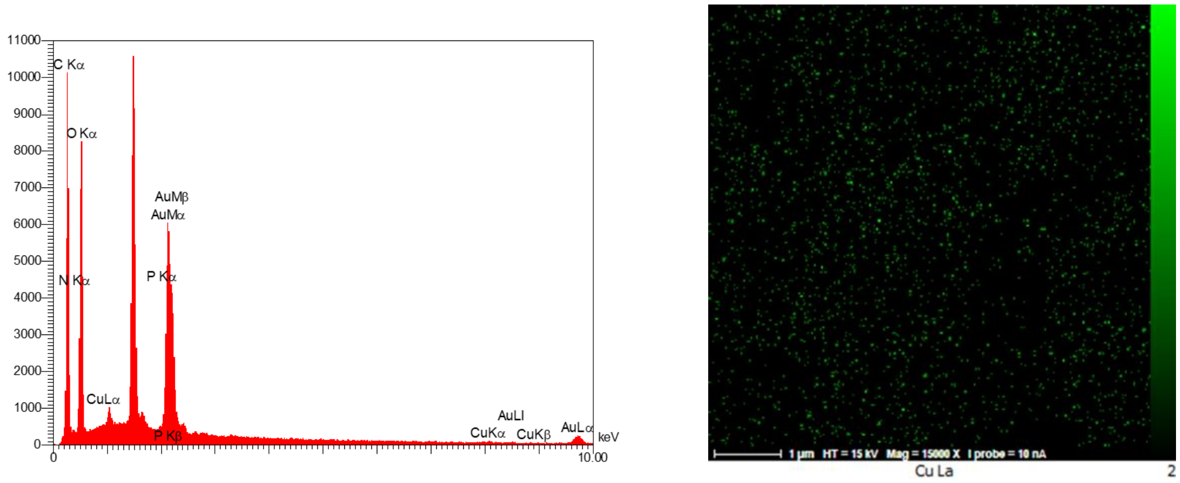


Fig. 2. ESEM images of nanocomposites; A. The produced CuO nanocomposite; B. The sizes of produced CuO nanoparticles; C. An overview of image A



A B

Fig. 3 A. EDX spectrum of Cu nanoparticles; B. Map of the dispersion of copper atoms on the polymer

Determination of antibacterial activity

A modified Kirby-Bauer disk diffusion method was used to assess the antibacterial effects of the nanocomposite and the acrylic polymer. The results shown in Fig. 4B indicate that microbial growth was observed under the surfaces treated with the acrylic polymer, although the growth was significantly reduced. In contrast, Fig. 4A shows no bacterial growth under the nanocomposites placed on the inoculated surfaces. It can therefore be concluded that the acrylic polymer has antibacterial properties, which are enhanced in the nanocomposite.

Determination of antimicrobial activity of nanocomposite and acrylic polymer

This method is used to assess the antimicrobial effect of materials combined with hydrophobic substances such as plastics, epoxy resins and other hard surfaces by determining the percentage reduction of microbial cells. The reduction of bacterial and fungal cells on the copper nanocomposite and acrylic polymer was presented in Table 1.

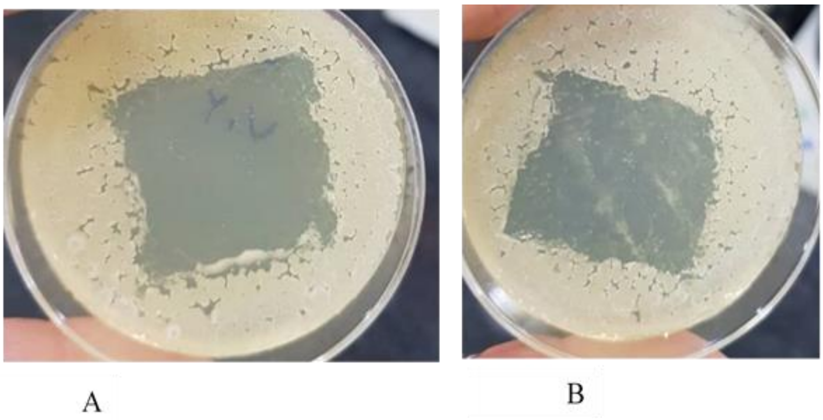


Fig. 4. A. No bacterial growth under the nanocomposite layer; B. Decreased bacterial growth under the acrylic polymer

Table 1. The CFU reduction of the bacterial and fungal cells on nanocomposite and acrylic polymer

Bacteria	**Initial load (0 h)**	**Nanocomposite (18-20 h)**	**Acrylic polymer (18-20 h)**
B. subtilis	2.8×10^{6}	2.1×10^{2}	2.2×10^{5}
Ps. aeruginosa	2.7×10^{6}	1.1×10^{2}	1.4×10^{5}
Fungi	**Initial load (0 h)**	**Nanocomposite (90-96h)**	**Acrylic polymer (90-96 h)**
A. niger	1.2×10^{5}	1.2×10^{3}	1.1×10^{4}
A. alternata	2.9×10^{5}	2.5×10^{3}	1.4×10^{4}
C. cladosporioides	2.7×10^{5}	2.5×10^{3}	2.3×10^{4}

As shown in Table 1, the polymer reduced the number of bacterial and fungal cells by one logarithm, while the nanocomposite showed the reduction of 2-4 logarithms. Furthermore, the decrease in bacterial and fungal cells can also be observed in Fig. 4B. The results demonstrate the superiority of the nanocomposite over the acrylic polymer.

Determination of the antimicrobial activity of the treated stone

The antimicrobial effect of the copper nanocomposite on stone samples was evaluated by measuring the CFU recovery of the treated samples. The results presented in Table 2 show the CFU recovery from the stone samples treated with the nanocomposite. The nanocomposite effectively reduced the number of microbial cells by 60%.

Biofilm formation on nanocomposites

An extensive biofilm was formed on acrylic polymer-coated glass surfaces, as shown in Fig. 5A. After 72 h of incubation, the entire surface was covered with a dense bacterial biofilm. In contrast, no biofilm was observed on glass surfaces coated with the copper nanocomposite during the same period (Fig. 5B). As shown in Fig. 5B, only a few scattered bacteria were visible on the surfaces, without significant colonization of bacteria on the glass. Lower magnification of biofilm and bacterial cells was shown in Fig.5C and D.

Table 2. The CFU recovery from stone samples treated by the nanocomposite and acrylic polymer

Treatments	Nanocomposite	Antimicrobial effect (%)
1st treatment	1.5 ± 0.18	60%
2nd treatment	1.8 ± 0.28	41%
3th treatment	1.4 ± 0.22	57%
Average	1.56 ± 0.22	52%

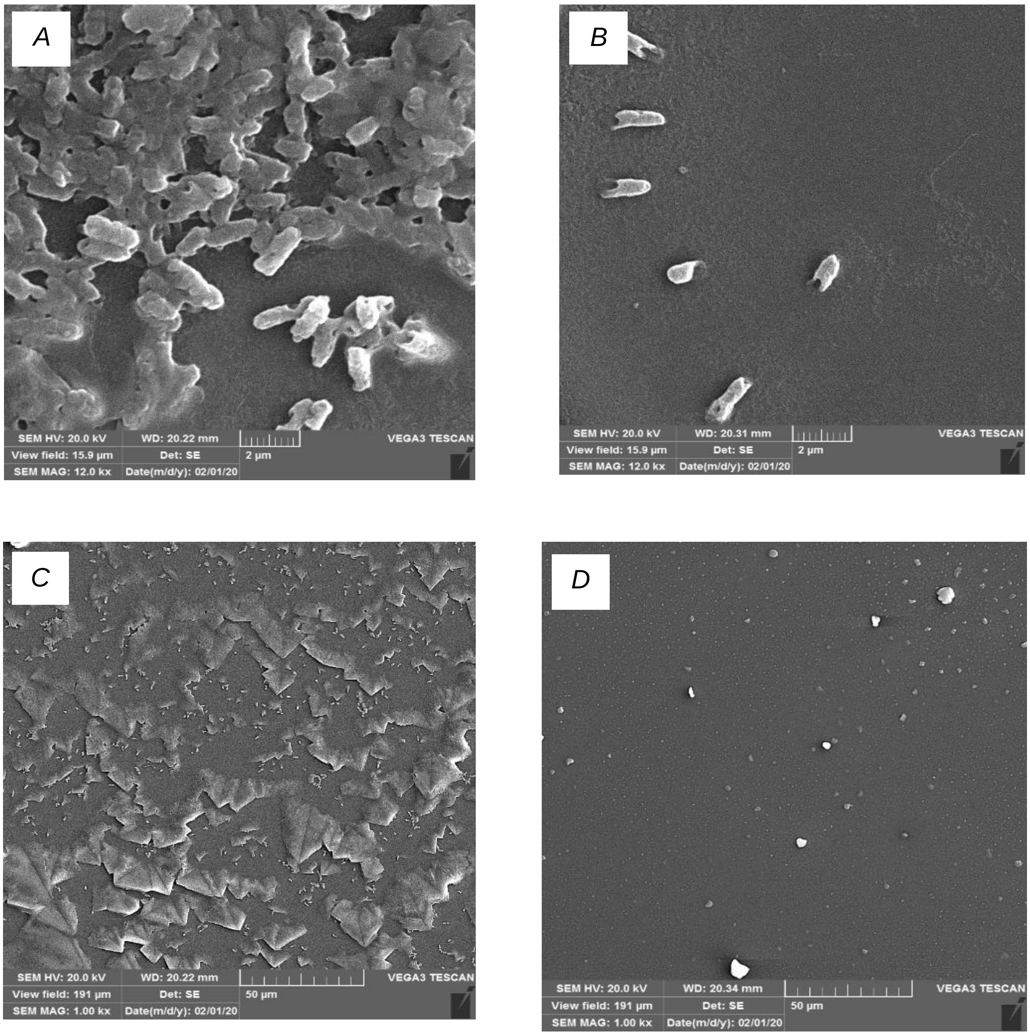


Fig. 5. Formed biofilm on glass surface; A. Ps. aeruginosa biofilm on acrylic polymer-coated glass surface after 72 h incubation; B. CuO nanocomposite-coated glass surface without formed biofilm after 72 h incubation; C. Biofilm of image A with lower magnification; D. Bacterial cells of image B at lower magnification

Physical properties of polymers and nanocomposites

Water is a key factor in the chemical and microbiological deterioration of historic monuments. Coatings and consolidants with hydrophobic properties minimize the penetration of water into the stone. As shown in Table 3, the nanocomposite not only provided better water repellency than the acrylic polymer, but also had less porosity.

Table 3. Physical properties of the coated stones by acrylic polymer and nanocomposite

Stone treatment	Water absorption (%)	Porosity (%)	Density (gm/cm^3)
Acrylic polymer	0.08%	0.19%	2.84
Cu nanocomposite	0.05%	0.13%	2.87

Discussion

Many researchers and conservators worldwide are focusing their efforts on the critical issue of deterioration of stone cultural heritage in outdoor environments (10, 30, 31). In an effort to improve the physicochemical properties and antibacterial activities of nanocomposites used for the consolidation and protection of historic and architectural stone, a novel bioengineered copper nanocomposite was developed through an *in situ* approach.

Since the 1960s, synthetic polymers have served as the primary means of consolidating cultural heritage objects, buildings and stone materials (32, 33). A large number of tests have been carried out to evaluate the chemical and physical stability of acrylics, demonstrating their general effectiveness in strengthening and protecting stone. However, few studies have investigated the susceptibility of these materials to biological degradation (34). Fungi are acknowledged as particularly destructive organisms that can affect the surfaces of stone monuments (35, 36). It has been reported that certain synthetic polymers used in paints can be contaminated by fungi, particularly genera such as *Rhizopus*sp. and *Aspergillus* sp. (37).

Biosynthesis and characterization of nanocomposites

Polymeric composites incorporating nanoparticles have introduced new opportunities for the development of conservation materials with enhanced hydrophobicity (4, 38) and antimicrobial properties (38-40).

Incorporation of nanoparticles into polymeric materials is often achieved by chemical methods like reduction or mixing of preformed nanoparticles with polymers, or by physical techniques such as sputtering and plasma deposition. These methods increase the synthesis time, cost and complexity of producing antimicrobial materials (10, 41). Therefore, there is a need to find a simple method to incorporate nanoparticles into polymers. Adnan *et al.* (2018) highlighted that the *in situ* precipitation method is a versatile technique for synthesizing nanoparticles. In this study, for the first time precipitation was used to directly produce polymer/bioengineered nanoparticle composites in a single step. The spherical nanoparticles were distinctly observed on the polymer, indicating that precipitation occurred adjacent to the polymer chains. If the CuNPs had precipitated in solution away from the polymer chains, their distribution throughout the polymer matrix would not have been uniform. However, as precipitation occurs close to the polymer chains, it helps to stabilize the formation of CuNPs and prevents aggregation. These results are consistent with previous research (42). Chalal *et al.* have sown that *in situ* polymerization allows for a uniform distribution of nanoparticles within the polymer matrix, as metal ions dissolve readily in aqueous or alcoholic media (43). A comparison of *in situ* and ex situ techniques for the preparation of polymer/TiO_2 nanocomposites showed that *in situ* synthesis is a rapid and efficient method for producing nanocomposites. The *in situ* method, which generates nanoparticles from precursors in a single process, results in well-formed nanocomposites (21). Adnan *et al.* also investigated the *in situ* production of hybrid inorganic-polymer nanocomposites and found that the *ex situ* technique leads to greater heterogeneity and potential damage to material properties, and separation of agglomerates formed during nanoparticle production is difficult due to the high viscosity of the polymer (20).

In summary, the *in situ* method is more suitable for the synthesis of nanoparticles due to the homogeneous dispersion within the polymer matrix

(20, 44). Therefore, in this study the *in situ* approach was used to synthesize the bioengineered copper nanocomposites to enhance the antibacterial properties of acrylic polymers.

Antibacterial activity of nanocomposites

The antimicrobial mechanisms of nanoparticles and nanocomposites operate through multiple pathways, including cell wall and plasma membrane disruption, inhibition of protein synthesis, interference with DNA replication and enhanced cellular oxidation (45, 46). Furthermore, nanoparticles demonstrate the ability to inhibit microbial proliferation by diffusing into the exopolysaccharide matrix of biofilms, down-regulating quorum sensing proteins and/or enhancing quorum quenching within biofilm architectures, subsequently leading to degradation of extracellular polymeric substances (EPS) and microbial mortality in natural environments. The antimicrobial efficacy of nanoparticles is controlled by various parameters, including particle dimensions, concentration, pH conditions, exposure time, morphology and surface modification (47, 48).

Copper, in particular, exhibits strong bactericidal activity when incorporated into materials (49, 50). While acrylic copolymers have limited antimicrobial properties against bacterial and fungal strains (51), studies have confirmed the antimicrobial efficacy of methacrylate polymers against standard bacterial strains, particularly *S. aureus* and *E. coli* (52). Our initial experimental results indicate that acrylic polymers exhibit antimicrobial properties, which are consistent with previous studies (9). The bactericidal mechanism of CuNPs primarily involves the generation of reactive oxygen species (ROS), which induce protein and DNA oxidation, lipid peroxidation and membrane deterioration (53). Despite their proven antimicrobial efficacy and extensive application in biomedical contexts, CuNPs remain underutilized in heritage conservation compared to alternative antibacterial agents such as TiO_2 or AgNPs (35). The inherent challenges of copper include its rapid oxidation and low toxicity. Nevertheless, recent investigations indicate that the integration of CuNPs with consolidants demonstrates significant efficacy in the treatment of stone surfaces colonized by various microorganisms, including bacteria, fungi, algae and lichens (54, 55). One notable study evaluated CuNPs in combination with three commercial consolidants and water repellents (Silo 111, Acrilico 30 and Estel 1000) on pre-cleaned substrates from the ancient site of Fiesole. The results showed that environmental conditions and substrate bio-receptivity significantly influenced microbial recolonization post-treatment. Although the materials demonstrated reduced colonization, they did not completely inhibit the regeneration of lichens and biofilms (55). Moreover, incorporation of NPs into commercial consolidant polymers (Primal AC33 and silicone polymer) resulted in a 61% to 68% reduction in the growth of *E. coli, S. parvulus* and *B. subtilis*. CuNPs showed significant antibacterial efficacy against bacterial and fungal strains isolated from the Saqqara necropolis (56). However, due to copper color changes, researchers are more likely to use AgNPs in heritage. The present investigation demonstrated that the nanocomposite had satisfactory antimicrobial and antibiofilm properties. Additionally, photocatalytic zinc oxide nanoparticles embedded in Paraloid B acrylic showed adequate bactericidal, self-protective and hydrophobic properties in marble surface protection (1). The inhibitory and self-protective properties of the polymer composite against fungi have previously been validated without surface color changes (10). These improvements in the physical properties of the polymer confirm the importance of nanoparticles in the production of effective nanocomposites, in line with previous research.

Conclusion

Using the *in situ* bioprocess, nanoparticles were synthesized within a polymer matrix, significantly enhancing its antibacterial properties. This method is both cost-effective and environmentally friendly. In addition to inhibiting microbial growth on ancient stones, the nanocomposite can also enhance the physical properties of the treated stones. The critical next stage is to carry out *in-situ* experiments and evaluate its effectiveness in natural environments. Long-term assessment of antimicrobial treatments remains of paramount importance. Interdisciplinary collaboration among microbiologists, chemists, materials scientists and conservation researchers is essential to ensure the sustainable preservation of cultural heritage monuments.

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Declaration of interests

The authors declare that they had no known competing financial interests or personal relationships that could have appeared to influence on the work reported in this paper.

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