

Research

Enhancing the cell viability and antibacterial properties of alginate-based composite layer by adding active particulates

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Abstract

Because of their exceptional biological, mechanical, and structural capabilities, polymer matrix composite materials are immensely appealing in the biomedical industry, particularly for surface activation of metallic implants and tissue engineering. The purpose of this research is to investigate the effect of adding 5% active metallic particles (Mg particles and Ag nanoparticles) to an alginate matrix on cell survival (fibroblast MG-36 cells), antibacterial activity, surface texture, roughness, and wettability. The incorporation of 5% Mg particles and Ag nanoparticles in polymeric matrix employed composite material with outstanding properties. The adding of Mg and Ag particles boosted wettability by decreasing the contact angle from 56.83° to 0°. The activity of *Candida albicans* and *Porphyromanoas gingivalis* was significantly reduced, particularly with Ag nanoparticles, which fight bacteria by blocking cell development and improving the antimicrobial properties of composite materials. The use of Ag nanoparticles to strengthen alginate resulted in higher fibroblast proliferation for MG-63 cell at the outer layer, which resulted in superior cell viability (24, 48, and 72). Meanwhile, due to Mg's high activity, the reverse effect was found when Mg particles were used, making the composite surface more active (enriched with Mg ions).

Article Highlights

- Enhancing the alginate characteristic by utilizing magnesium particles with nano silver producing bio active composite, since Silver – magnesium now day has great deal in scientific research due to their biocompatibility and structural familiar close to human tissue.
- Obtaining new particular reinforcement configuration (nano –silver covered Mg particles) by with using simple preparation technique (casting), preventing formation of hydrogen bubbles in the surrounding tissue.
- Using nano silver with Mg particles as reinforcement maintain the viability of cells in an acceptable range, the cell viability to minimum value at 24hr (62.32%) but at 72hr silver nano particles lead to rise the viability up to 70% this was an indication to the positive effect of using dual type of reinforcement on the cell viability.

Keywords Alginate matrix · Magnesium particles · Nano silver · Fibroblast MG-36 cells · *Candida albicans* · *Porphyromanoas gingivalis*

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1 Introduction

Bio-composites are composites that can be used in bioengineering. The characteristics of elements are preserved in the composite. For instance, they do not melt or integrate entirely into each other even though they operate in concert. Most of the active tissues, such as dentin, bone, skin, and cartilage, are basically composites [1]. An artificial composite is made up of two basic phases: a strengthening component, such as fiber or particle, and a condensable known as a matrix. The main aim for developing bio-composites is to acquire various mechanical and biological features by changing the form and dissemination of the reinforcing elements at different stages in the composites and thus maximize the condition and performance of the implantable implants and their relationship with the external tissues. Several factors led to the growth of composite materials. The stiffness imbalance between bones and implants affects the load distribution among bones and implants in orthopedics. As the level of stress borne by them is exactly proportional to their stiffness, the bone is under loaded in comparison to the implant. Wolff's law of stress-related bone remodeling states that [2]. This could result in decreased bone density and a modified bone structure. It may also affect the recovery of shattered bones and increase the risk of bone fracture after replacement of the osteosynthesis implant, such as a bone plate, in osteosynthesis. Fitting the stiffness of the implant to that of the host tissues may prevent such adverse influences and provide optimal care for bone tissue regeneration. Particle-reinforced polymers, or polymer composite materials, have been suggested for various orthopedic uses due to their low elastic modulus and great strength. Another advantage of composite materials is that the features and shape of an implant may be altered and adjusted to meet the mechanical and physiological requirements of the host tissues by altering the volume fractions and confining the total organization of the reinforcing phase. As a result, composite materials have a much superior possibility of structural biocompatibility than homogeneous monolithic materials. Meanwhile, natural materials, such as adhesive, rubber, wood, and tissues, were initially chosen, and ongoing efforts by biomaterial scientists around the world have led to artificial materials, such as metals, ceramics, plastics, and glass, becoming entirely appropriate for biomaterial building. Nowadays, which biomaterials could be permitted or refused by the body is fairly clear. Furthermore, synergistic function while having suitable contact between composite biomaterials and body tissues is virtually completely understood compared with using non-composite biomaterials [3].

Natural materials are still commonly used in the repair and regeneration of human tissues because they are compatible and, more significantly, disposable inside the body. Alginate is a biological material that is frequently employed in the repair and regeneration of human tissue, especially for wound management [4]. Because it is used in human food applications, alginate is considered safe for biological devices. Alginate is also biodegradable because, when crosslinked with monovalent positive ions found in body fluids, it suffered from gradual dissolution in the simulated bodily fluids. The pace of alginate dissolution could be regulated by oxidation, leading to decreased alginate molecular weight [5, 6]. Finally, the ability of alginate to form gel is the major reason for its use in tissue engineering and wound healing treatment. Alginate was recently combined with different materials to form alginate composites, meeting additional biomedical needs [7–9] and increasing soft-tissue formation and repair [10, 11]. Alginate is a polymer found in algal cell walls as well as *Pseudomonas* sp. and *Azotobacter* sp. bacterial capsules. The presence of alginate in the cell walls of brown algae (also known as brown seaweeds) provides the algae with flexibility and strength, also act as a barrier against potential harm when the algae are subjected to powerful seawater waves [12]. Alginates are composed of 1,4-linked-L-guluronic acid (G) and 1,4-linked-D-mannuronic acid (M) residues that combine to create blocks of repeated M, repeated G, and alternating G and M residues [13]. The natural resources used to manufacture alginate determine the sequence and quantity of G and M residues. Furthermore, the alginates produced from *L. hyperborean* have a G concentration of 60%, whereas those derived from other commercialized microalgae have a G value of 14–31%. Only the carboxylic groups in G residues were proposed to crosslink with divalent cations, such as Mg^{2+} and Ca^{2+} , to generate hydrogels, resulting in stiffer hydrogel produced from alginates with high G concentration and soft elastic hydrogel alginates obtained from high M concentration [14]. Despite the fact that alginate is considered a non-toxic, biocompatible, and non-immunogenic material, studies have shown that alginate with a high G concentration is less immunogenic than alginate with a high M concentration [15]. To enhance the physical properties of alginate, other compounds have been combined with it to create an alginate composite. Alginate composites are made by mixing natural polymers like gelatin, chitosan, and collagen with synthetic polymers like polypyrrole and polylactide, as well as inorganic chemicals like hydroxyapatite and tetraethylorthosilicate [16, 17]. Incorporation of various materials, such as bioactive glass, bio-ceramics, inorganic nano-additives, and inorganic carbon-based ceramics, has also been utilized by previous researcher [18, 19]. Several alginate composites

Table 1 Alginate and reinforcing metal properties

Materials	Properties			
	Shape	Color	Size	Purity %
Alginate	Powder	White	100 µm	95.6
Magnesium (Mg)	Powder	Gray	250 µm	98.9
Silver (Ag)	Powder	Dark gray	70 nm	99.5

Table 2 Compositions of composite layer samples

Sample	Composition
1	Alginate (Pure)
2	Matrix (Alginate) + 5% vol. of Mg
3	Matrix (Alginate) + 5% vol. of Ag
4	Matrix (Alginate) + 5% vol. of (Mg + Ag)

have showed remarkable results and are commercially available, particularly for tissue engineering applications [20]. Meanwhile, others are still undergoing development. The current study aimed to improve the bioactivity of metallic implants by creating an actively biocompatible composite layer at the implant surface, accomplished by utilizing an alginate film strengthened by 5 vol.% metallic particles (Mg particles and Ag nanoparticles) [21]. For pure alginate or alginate in form of composite various biomedical applications involved this type of biological substance, into several forms such as hydrogel, bead, 3D printed material or fiber, this was according to specific demand of applications. Treatments for bone defects normally include natural grafting from human donor (auto- and allograft) and from other species (xenograft) [22]. Preparation of graft materials can also be utilized and numerous studies had been conducted to replace the natural grafting that has wide range of limitations such a availability, risks associated with harvesting either from human or other species and requires additional surgeries that may cause infection to both the receivers and donors [23]. Alginate is one of the materials that were extensively studied for synthetic bone grafting. It can act as scaffolding materials for bone construction and can be used to deliver cells for bone tissue regeneration. It was demonstrated that alginate also supported the differentiation of murine embryonic stem cells (mESCs) to form bone cells. However, alginate alone is incapable for load-bearing orthopaedic applications because it does not have mechanical strength. Thus, many researches develop 'stronger' alginate composites for bone applications [24]. Biodegradation/ higher corrosion rate of Mg and its alloys causes formation of hydrogen bubbles in the surrounding tissue, which may delay healing or even induce necrosis of tissue. OH⁻ anions generated in the corrosion reaction increase the pH of the fluid around Mg surfaces, which influences surrounding cellular functions and decreases cytocompatibility. The work also focused on preventing formation of hydrogen bubbles in the surrounding tissue by using other size scale and type of active metallic particles (silver) in nano scale, to cover the Mg micro particles get a combination of antimicrobial active nano cover on Mg particles in alginate matrix.

2 Materials and methods

A polymer matrix composite film was prepared using sodium alginate, which is reinforced by two different types of metallic particles at 5 vol.% (Mg particles and Ag nanoparticles). These types of metallic materials as reinforcing particles to produce the active biocompatible composite film were utilized to enhance the cell viability and antimicrobial behavior of hole implant materials used in hard tissue applications. The properties of alginate and reinforcing metal powders are summarized in Table 1.

Metallic reinforcing particles were used in different particle sizes. Mg was used on a micro-scale due to high corrosion rate and activity, whereas Ag was utilized on a nanoscale to enhance the surface area resulting in the increase in the microbial behavior of the composite material. The casting technique was used to prepare the composite film samples. The reinforcing particles were added at 5 vol.% to the alginate matrix, as shown in Table 2, which clarifies the total types of composite samples. Sodium alginate powder was dissolved overnight at room temperature with stirring at (600 rpm) without vacuum by applying de-ionized water as solvent to prepare solution with concentration of 5% (w/v). Then, the weighted reinforcing particles were added at 5% vol. For the matrix solution, good mixing was conducted using a

Fig. 1 FESEM morphology of pure alginate at 5000 x

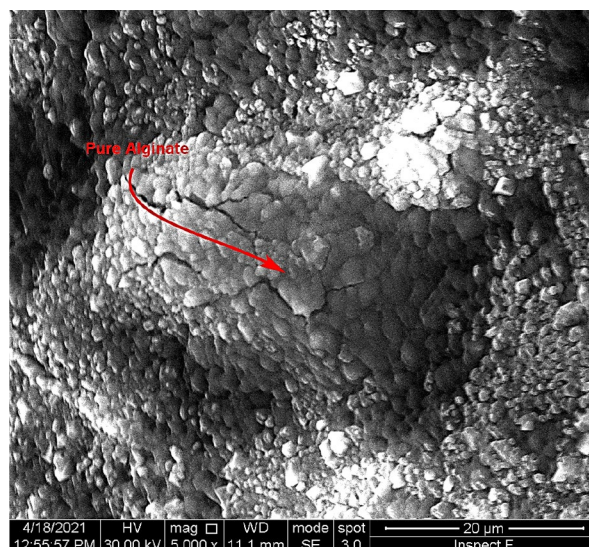
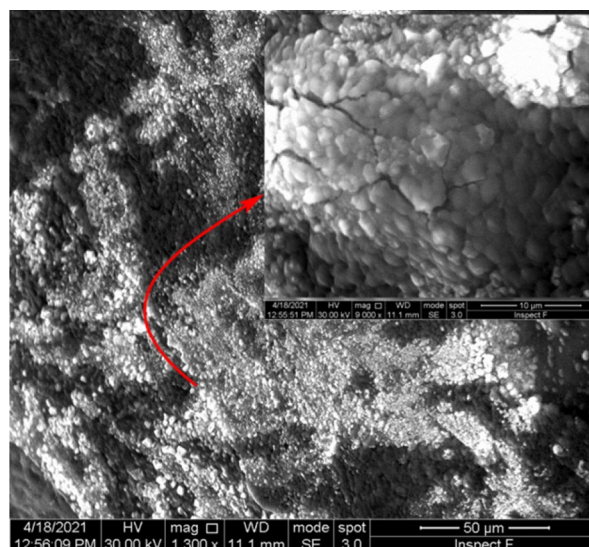


Fig. 2 FESEM morphology of pure alginate at 1300 x



magnetic starter for 2 h to produce a homogenous solution, resulting in high-quality composite film. The solution was then poured onto the dye until drying naturally at room temperature. The drying time was approximately 48 h. Finally, the composite film was removed from the dyes and characterized.

In this work, many characterization approaches were used. Field Emission Scanning Electron Microscopy (FESEM) and Fourier transform infrared spectroscopy (FTIR) were used to assess the structural and morphologic features of all composite films. Wettability, antibacterial behavior (drug sensitivity test by Kirby-Bauer method), and in vitro MTT assay were performed to assess the overall biological properties of the prepared composite layers.

3 Results and discussion

3.1 Field emission scanning electron microscopy (FESEM)

FESEM was employed to investigate the microstructures of composite film samples. The photos provided a comprehensive view of the samples' surfaces, elucidating the influence of metallic particle addition (Mg particles and Ag nanoparticles) on surface texture and roughness. Figures 1 and 2 depict the microstructure of a pure alginate matrix without any additions. A homogeneous and smooth surface texture was found, suggesting that alginate dissolution

Fig. 3 FESEM morphology of alginate matrix/magnesium particles at 2500 x

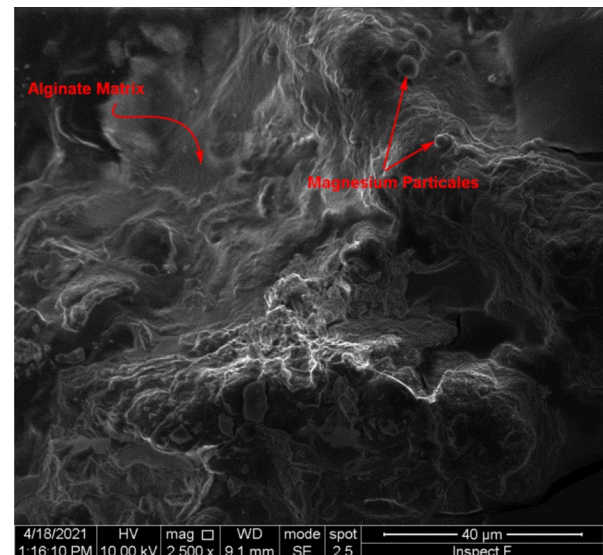
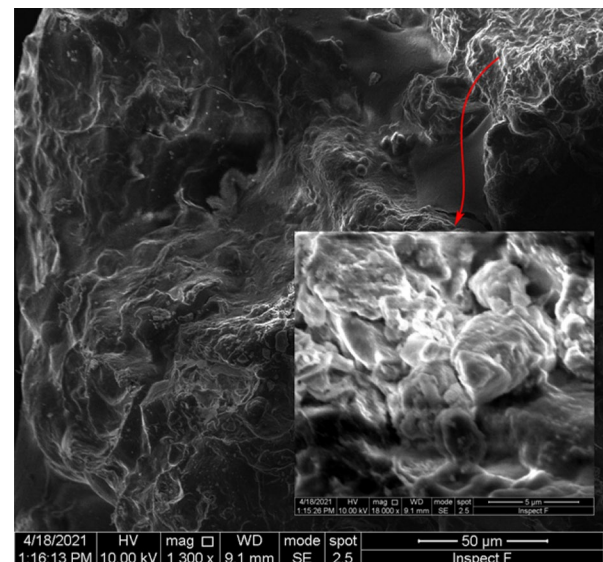


Fig. 4 FESEM morphology of alginate matrix/magnesium particles at 1300 x



and mixing were effective. With the addition of 5% Mg to alginate, relatively inhomogeneous distribution of Mg additions in the alginate matrix was found, imparting a heterogeneous texture to the outer surface and enhanced surface roughness, as shown in Figs. 3 and 4. Mg particles caused a progressively roughened surface and morphological alteration, although the porous nature of the composites was preserved. Figures 5 and 6 depict the microstructure of an alginate matrix containing 5% Ag nanoparticles. Because of the Ag nanoparticle impact, the surface texture was entirely altered, with an acceptable decreasing in the roughness as compared with composite layers reinforced with Mg additions. As seen in Figs. 7 and 8, the inclusion of Mg particles and Ag nanoparticles caused significant alterations in all surface textures. Because of the considerable variances in particle size and surface charge, the Ag nanoparticle overlapped on the surface of the Mg inclusions in the alginate matrix, making composite surface rougher with preferred uniformity. This served in the improvement the entire surface characteristics. This behavior may be attributed to large surface area of silver nano-particles, which caused the upraising the attraction forces and enhanced the precipitation of silver nano-particles on magnesium outer layers. Thus, this effect resulting in a desirable mixture of particulate materials as reinforcing phase with a greater dual influence in optimizing the overall properties of the prepared composites [25]. Using two metallic additions with diverse sizes assisted in optimizing the combination of them in the organic alginate matrix.

Fig. 5 FESEM morphology of alginate matrix/Ag particles at 2500 x

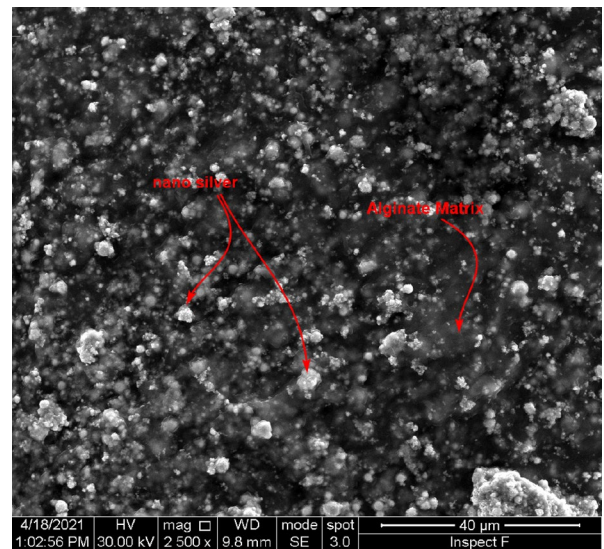


Fig. 6 FESEM morphology of alginate matrix/Ag particles at 5000 x

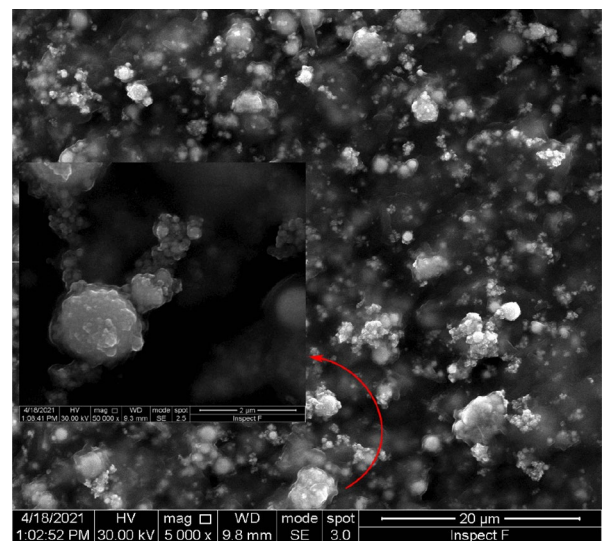


Fig. 7 FESEM morphology of alginate matrix/Mg + Ag particles at 2000 x

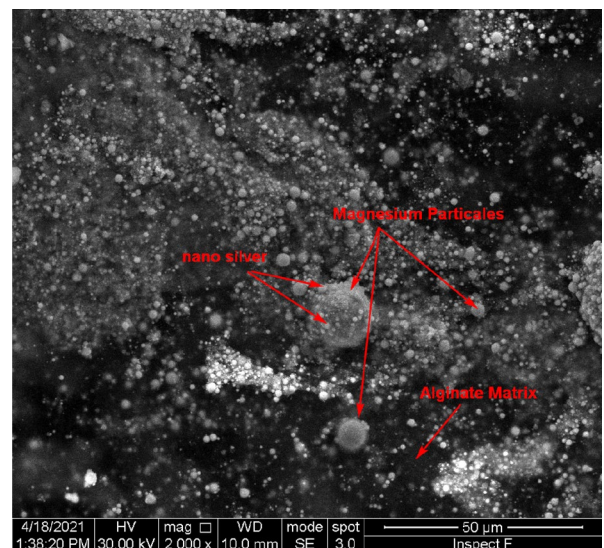
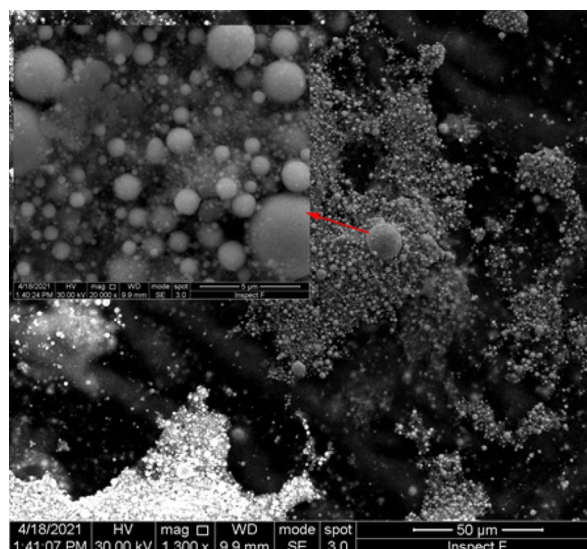


Fig. 8 FESEM morphology of alginate matrix/Mg + Ag particles at 1300 x



3.2 Fourier transformation infrared spectroscopy (FTIR)

FTIR spectrophotometer was utilized to analyze and compare the functional group produced from pure alginate matrix film sample and the alginate matrix with different metallic particles (Mg particles and Ag nanoparticles). Figure 9 shows the FTIR spectra of sample (1) for pure alginate matrix. The spectrum presented many strong bands of alginate hydrogels. The spectra of sodium-alginate films provided a broad transmission band at 3284 cm^{-1} , which was due to the OH group's expanding vibration band and the -CH vibration bands at 2899 cm^{-1} . The apparent bands at 1600 and 1405 cm^{-1} were assigned to the asymmetric and symmetric stretching vibrations of COO- groups, respectively, and are unique to ionic bonding. The addition of metallic particles (5 vol.% pure Mg) created a new band of composite that appeared in the FTIR spectrum, as shown in Fig. 10, especially at 2360 cm^{-1} . The existence of Mg in the alginate polymeric matrix caused the development of this band. Figure 11 depicts the composition of the alginate framework containing 5% Ag nanoparticles. The presence of Ag led to a reduction in the intensity of all alginate strong bands especially a large transmission band like (O-H) band at 3284.97 cm^{-1} , (C=O) band at 1600.40 cm^{-1} , and (C-H) band at 1020.14 cm^{-1} . The strong decreasing in (O-H) band intensity at 3207.66 cm^{-1} in this Figure rather than alginate bands was attribute to the nano silver effect,

Fig. 9 FTIR spectra of the sample (1) for pure alginate

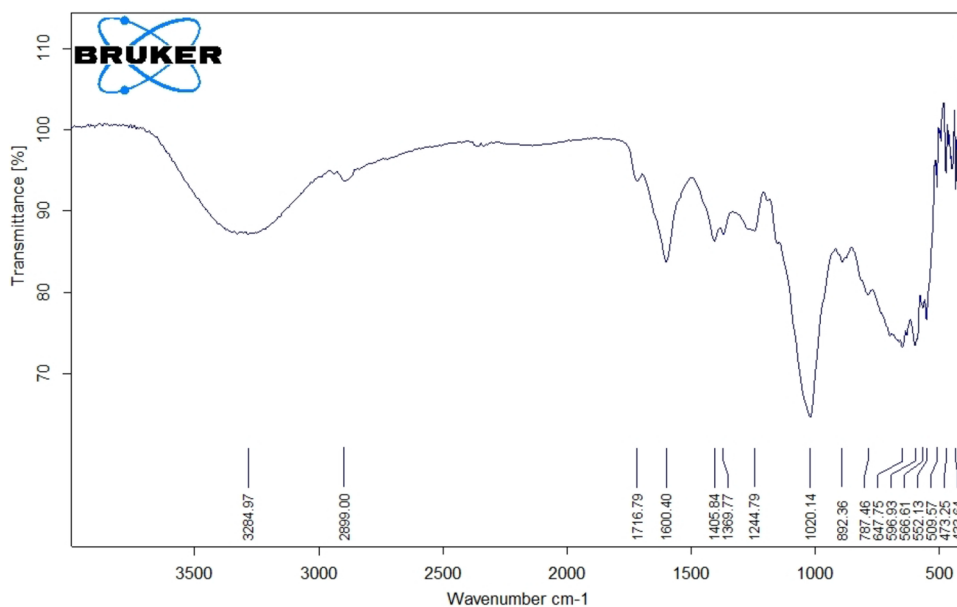


Fig. 10 FTIR spectra of the sample (2) for pure magnesium particles in alginate matrix

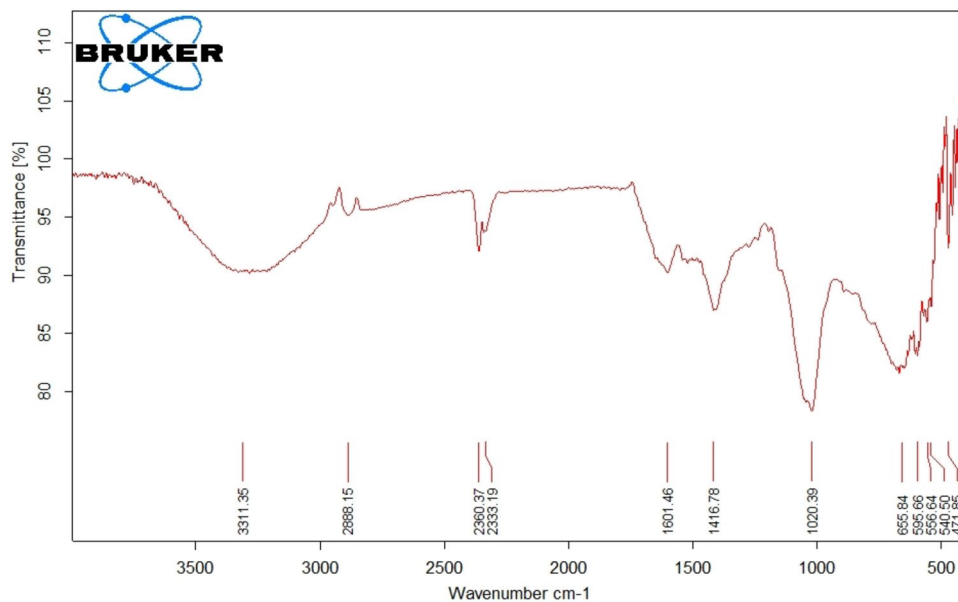
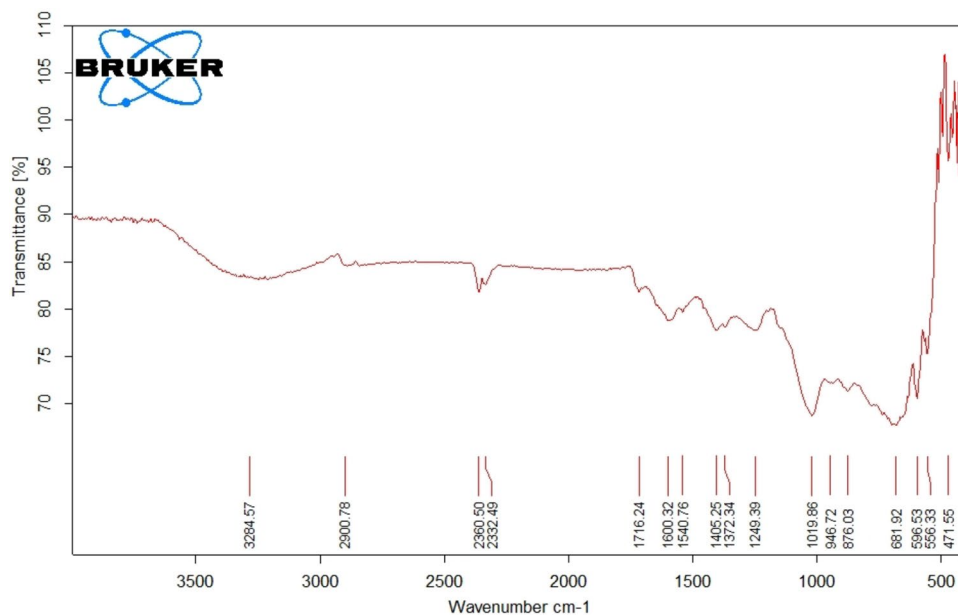


Fig. 11 FTIR spectra of the sample (3) for nanosilver in alginate matrix



which tend to absorb hydration of alginate polymer matrix. The combination of reinforcements (Mg and nano Ag) also resulted in a considerable decrease in band intensity due to the dual effect of reinforced metallic particles on the composition of the alginate matrix as shown in Fig. 12. Finally, no other new peaks appear for all composite samples as mentioned in Fig. 13 of collective FTIR samples spectra, this gives good indication of improving the miscibility state for all composite samples [26].

3.3 Contact angle measurement (WCA)

Water contact angle (WCA) was applied to estimate surface wettability by determining how many water droplets could reappear on a surface. Figure 14 shows WCA of all samples in order to demonstrate the influence of different metallic additions on the composite wettability.

Figure 15 represents WCA of control sample (pure alginate). The surface displayed a slight WCA of roughly 56.83°, indicating that it was hydrophilic. The adding of 5 vol.% Mg to the matrix caused reduction in WCA to 45.64°, as seen in

Fig. 12 FTIR spectra of the sample (4) for nanosilver and magnesium particles in alginate matrix

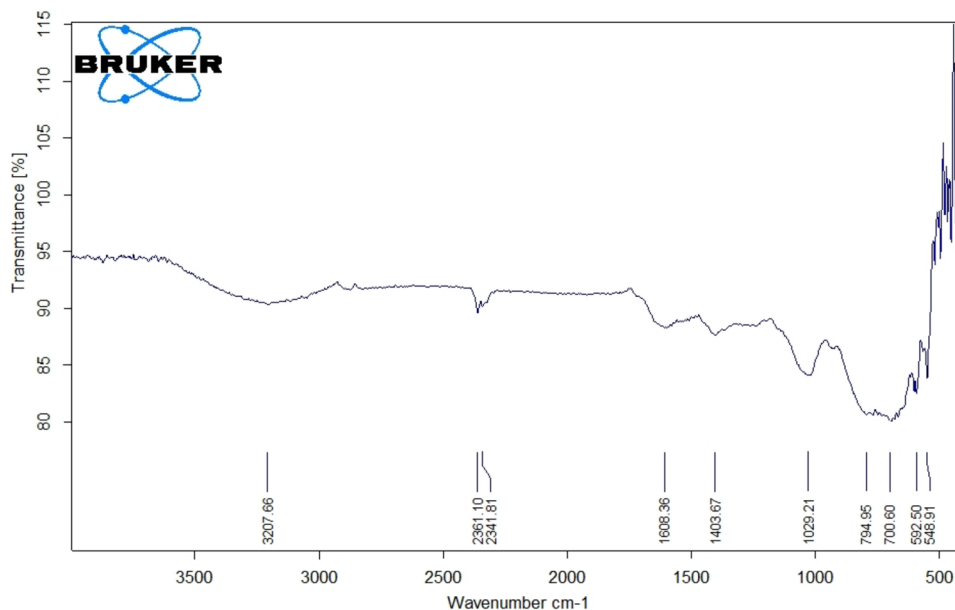
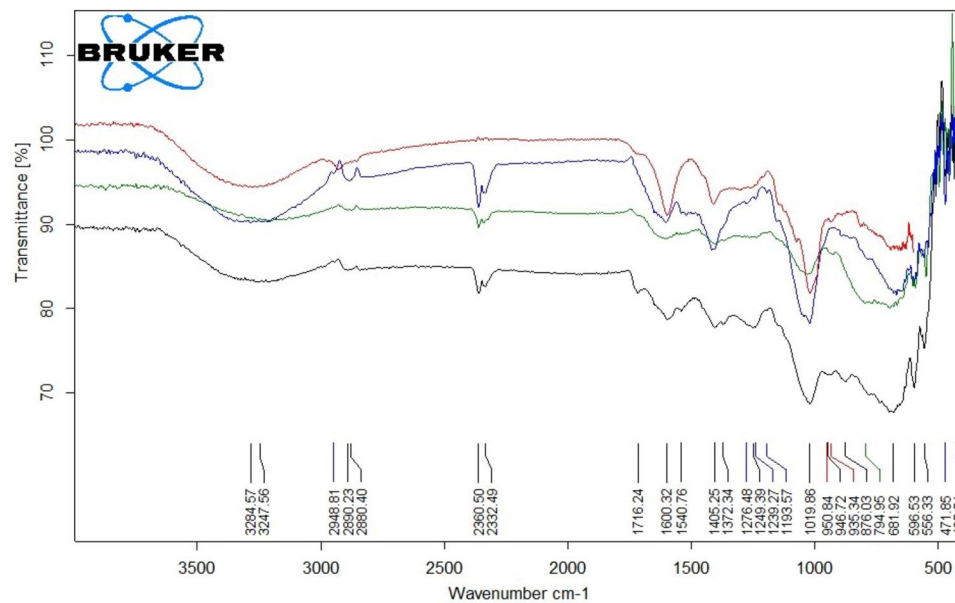


Fig. 13 Collective FTIR spectra of all samples



C:\OPUS_7.0.129\Meas\2021\Dr Fatima\Pure.1	Pure	Instrument type and / or accessory	06/04/2021
C:\OPUS_7.0.129\Meas\2021\Dr Fatima\Mg.0	Mg	Instrument type and / or accessory	16/03/2021
C:\OPUS_7.0.129\Meas\2021\Dr Fatima\Silver.0	Silver	Instrument type and / or accessory	16/03/2021
C:\OPUS_7.0.129\Meas\2021\Dr Fatima\Mg + Silver.0	Mg + Silver	Instrument type and / or accessory	16/03/2021

Fig. 16, since Mg alloy is hydrophilic with a water contact angle (θ) of $\sim 45^\circ$ due to its surface oxide layer. As a result, adding this percentage increased WCA of composite materials and made the sample with more hydrophilic surface. Figure 17 depicts the WCA of an alginate matrix containing 5% nano silver. Because of the rise in contact angle to 62.68° caused by the addition of nanoparticles, the surface's hydrophilicity visibly reduced. The nano reinforcement effect resulted in excellent and extraordinary interlocking between the polymer matrix and reinforcing particles, resulting in roughness reduction and improved surface uniformity. As shown in Fig. 18, the inclusion of two types of metallic particles in the polymer matrix resulted in a superior hydrophilicity with WCA of 0° . during the testing of sample (Mg particles with nano-silver in alginate matrix) the water droplet completely absorbed at sample surface with no contact angle (0), this is due to the formation of new configuration of reinforcing metallic particles (Mg full covered by nano silver) as illustrated in FESEM image, Fig. 8. Nano—silver covering lead to increasing metallic particulate surface area that rising the surface

Fig. 14 Water Contact angle of prepared samples

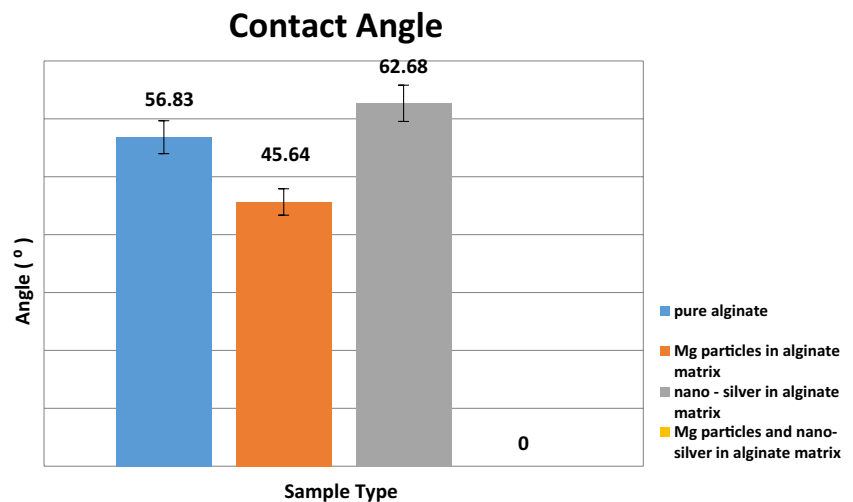


Fig. 15 WCA of pure alginate (Control sample)

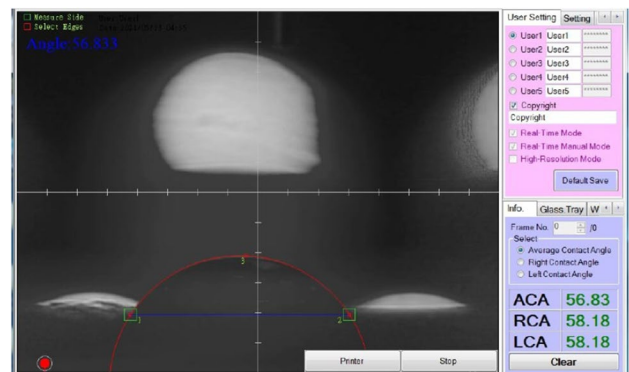
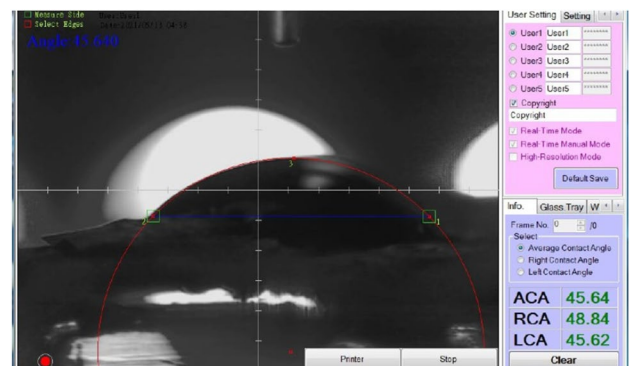


Fig. 16 WCA of alginate/Mg particles composite



hydrophobicity of whole composite, since the composite sample containing only nano—silver reinforcement had the higher contact angle value (62.68°) this is another indication of nano-silver effect on the wettability composite surface [27].

3.4 Antibacterial activity test

The antibacterial behavior of the composite material with different metallic particulate reinforcements (Mg particles and Ag nanoparticles) was determined using two types of bacteria, *Candida albicans* and *Porphyromanoas gingivalis*. The Kirby-Bauer approach conducted to assess the drug sensitivity. In this test, the bacteria colonies were grown removing a portion of them and soaking it in NaCl medium till the suspension reached 0.5 Mcf. Then, they transformed into a heated

Fig. 17 WCA of alginate/Ag particles composite

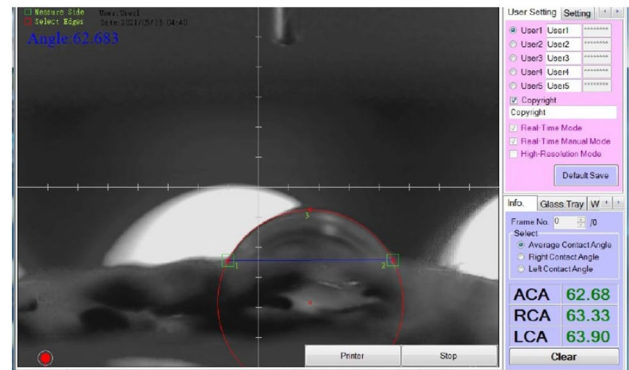


Fig. 18 WCA of alginate/Mg + Ag particles composite



Fig. 19 Inhibition zone of *Candida albicans* of pure magnesium particles in alginate matrix



plate in the center of agar. At 37 °C, the plates were incubated for 24 h. Following that, the resistance halos (the zone of inhibition) measurements in millimeters by utilizing a ruler as illustrated in Figs. 19, 20, 21, 22, 23, 24. The results demonstrated that adding metallic particles as reinforcing elements to the alginate polymer matrix improved the antibacterial behavior of complete composites by restricting the activity of bacterial proliferation and growth at the composite's surface. Figure 25 depicts the inhibitory zone diameter (mm) for composite material samples. Because of the nanoscale action of metallic particles, the addition of Ag nanoparticles to the alginate matrix resulted in a wide inhibitory zone with a diameter of roughly 32 mm for *C. albicans* and 16 mm for *P. gingivalis*. The micro metallic particles attacked the bacteria's cell walls, inhibiting the biological process and contributing to the composite's strong antibacterial activity. Meanwhile, due of the vast disparity in particle size between metallic reinforcement and *P. gingivalis* cells, the usage of

Fig. 20 Inhibition zone of *Porphyromonas gingivalis* of pure magnesium particles in alginate matrix



Fig. 21 Inhibition zone of nano-silver and magnesium particles in alginate matrix against *Candida albicans*

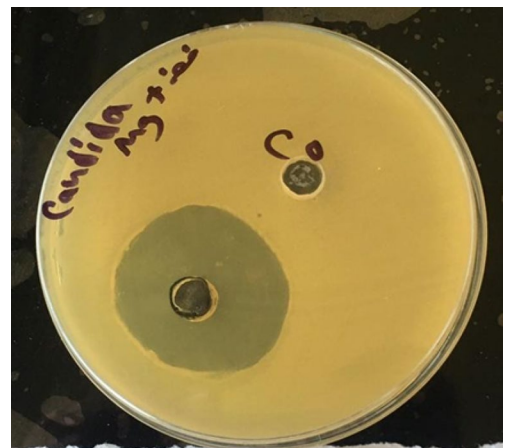
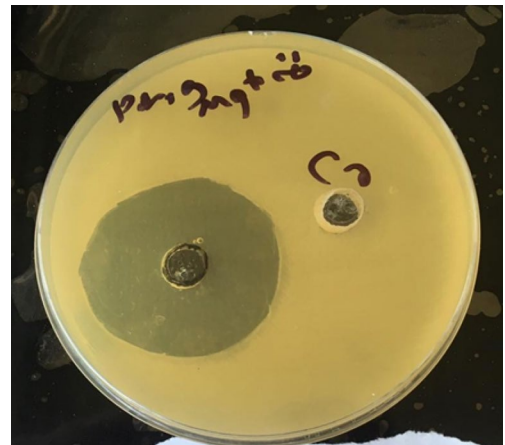


Fig. 22 Inhibition zone of nano-silver and magnesium particles in alginate matrix against *Porphyromonas gingivalis*



big metallic Mg particles had substantially less of an effect on *C. albicans*, with a diameter of 19 mm, and no effect on *P. gingivalis*. Mg is also a very active metal with a high corrosion rate.

The corrosion of active metallic reinforcement provided a large amount of oxygen in the composite material, leading to enhancement in the biological processes of bacteria at the surface and lowering of the antibacterial behavior of the composite. Two different types of metallic particles with different particle sizes were used to increase the antibacterial behavior of the composite material, as shown in Fig. 25. An increase in the diameter of the inhibition zone at 33 mm for *C. albicans* and 40 mm for *P. gingivalis* was observed this was due to the dual effect of

Fig. 23 Zone of Inhibition of nano-silver/alginate matrix against *Candida albicans*

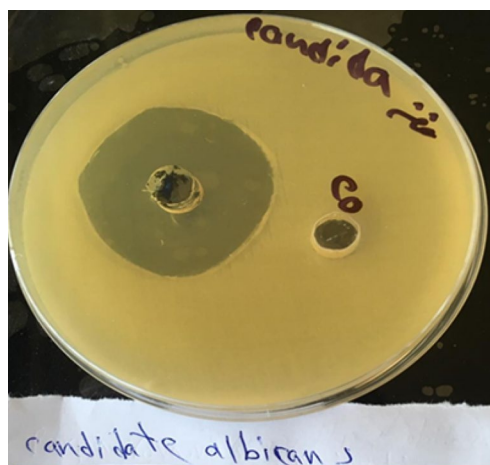


Fig. 24 Zone of Inhibition of nano-silver/alginate matrix against *Porphyromonas gingivalis*

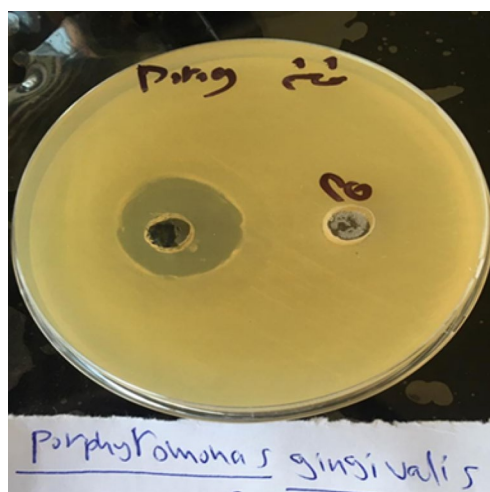
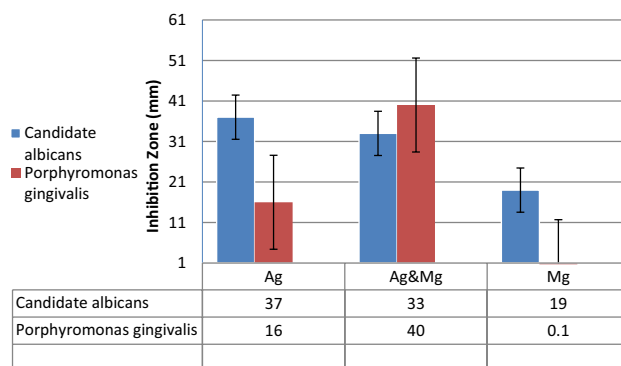
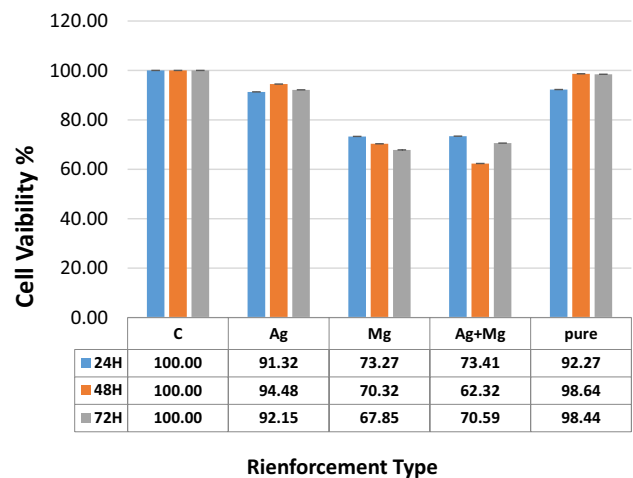


Fig. 25 Antibacterial activity of composite material containing particulate metallic reinforcements (Mg particles and Ag nanoparticles)



metallic particles on the cell wall of both bacteria. Furthermore, as mentioned in the microstructural analysis, the precipitation of silver nanoparticles at the surface of Mg particles in the alginate phase, due to substantial variations in particle size and surface charge, making the composite’s outer layer rougher with preferable homogeneity, which helped enhance the hole surface properties; thus, the silver nanoparticles have the chief effect on killing the bacteria and improving antimicrobial activity [27].

Fig. 26 MTT assay of alginate with reinforcing materials



3.5 MTT assay (cell viability)

The MTT test employed to assess the 3D cell growth of MG-63 cell line cultured after 24, 48, and 72 h. MG-63 cell lines were cultured in medium with 10% bovine blood serum + 1% penicillin–streptomycin antibiotic. After that, cells were harvested using trypsin EDTA-0.25% enzyme, and the flask floor (80%) filled with cells. For 5 min, the centrifuging of cell suspension was performed at 1200 rpm. After centrifuging, the remaining of the cells were removed and the sediment cells settled in fresh growth media. Pure alginate showed a high degree of biocompatibility (cell viability) very close to the control sample in 24, 48, and 72 h of exposure periods. Because alginate is a biological and biodegradable polymer, it dissolves progressively inside the organs when the crosslinking agents are released and then undergoes an exchange reaction with the monovalent positive ions (cations) widespread in human fluids. Thus, the inclusion of active particles as reinforcing phases to the alginate main phase had a significant influence on the cell's survival. Figure 26 illustrates the MTT test of prepared composites. Using Ag nanoparticles to reinforce alginate resulted in improved proliferation for MG-63 cell at the surface, resulting in reasonable cell viability after periods of 24, 48, and 72 h of exposure. This may be attributed to Ag's impact on dropping the degradation rate. By contrast, the opposite effect was observed when using the Mg particles due to the high activity of Mg, making the composite surface more active (enriched with Mg ions) and increasing the degradation rate as the degradation in body fluid is more complex than in natural surroundings. The reason is because the dilapidation rate is influenced by various influences, such as protein and pH value change. Such influence prevents the adhesion of MG-36 cells, with a reduction in the proliferation process, which affects cell viability. The addition of Ag nanoparticles to Mg particles as a reinforcing material minimized the effect of Mg ions and improved cell viability, nano silver acts to maintain the viability of cells in an acceptable range, the cell viability of this sample at 24 h reach to minimum value (62.32%) but at 72 h silver nano particles lead to rise the viability up to 70% this was an indication to the positive effect of using dual type of reinforcement on the cell viability as shown in Fig. 26 [15].

4 Conclusion

Adding particulate reinforcing materials with differ particle sizes to the alginate improved composite characteristics by boosting wettability and decreasing contact angle from 56.83° to 0°. *C. albicans* and *P. gingivalis* activity was reduced, particularly when Ag nanoparticles were used, which attacked the bacteria, blocking cell proliferation and improving the antimicrobial action of the composite material. Using Ag nanoparticles to reinforce alginate resulted in increased MG-63 cell proliferation leading to very extraordinary cell viability values (24, 48, and 72 h), whereas using Mg particles resulted in the opposite effect due to the elevated activity of Mg, making the composite's surface more active (enriched with Mg ions).

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Author contributions FJA: editing, revising, supposed the tests, and supervised the manuscript work. QAH original draft authoring, data collection and preparation. NKF: edited the article. In addition, all authors reviewed the manuscript.

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Data availability The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Declarations

Ethics approval Not applied.

Consent to participate Consent was obtained from all the authors included in this article.

Consent for publication The authors agree to publish.

Competing interests The authors have declared that no competing interests exist.

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