NANO-ENHANCED WOUND CARE: MANDARIN OIL-COATED AgNPs IN WOUND DRESSINGS

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The growing concern over antibiotic-resistant bacteria in wound infections has driven researchers to develop innovative solutions. Nanotechnology represents an effective and modern solution to overcoming wound infections that hinder healing. Developing a hydrogel with antimicrobial agents in the form of nanoparticles leads to a composite dressing that includes the characteristics required for successful healing, reassuring the properties of each material used and thus ensuring the action of all in one place. The present work is centralized on developing a hydrogel dressing made from polyvinyl alcohol and alginate, including silver nanoparticles and antimicrobial mandarin oil. The samples were analyzed using physicochemical techniques, including FT-IR, XRD, SEM, TEM, and biological tests, to assess their antimicrobial activity and biocompatibility. The results obtained confirm that this solution developed to stimulate the natural healing process through its main activity against the development of biofilms presents an effective approach due to the architecture of the hydrogel, which allows the release of silver nanoparticles covered

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with mandarin oil as well as due to its structure which fulfills many of the necessary conditions for the healing of an infected wound.

Keywords: wound dressing, silver nanoparticles, mandarin essential oil, alginate, polyvinyl alcohol

1. Introduction

It is well-known worldwide that dressings represent an important segment of the medical and pharmaceutical market in terms of wound care. Following the studies regarding the healing of wounds, it was demonstrated using a dressing that maintains a consistent level of moisture can significantly accelerate and enhance the healing process, promoting faster and more effective recovery. Over the past two decades, the introduction of new wound dressings has led to a steady decline in the number of patients presenting with wound infections and complications, as these innovative products have helped to reduce the occurrence of such issues. [1-5].

In recent years, nanostructured materials technology has led to major interest for scientists since different types of metallic (gold, silver) or oxidic (compounds of copper, zinc, titanium, magnesium) nanomaterials present attractive properties and versatile functionalities. Silver nanoparticles have been evaluated as the most effective in bioactive dressings with applications in anti-infective therapy due to their demonstrated antimicrobial effectiveness against bacteria, viruses, and other microorganisms. Silver has been used since ancient times in various activities, being more and more studied and advanced due to its physical, chemical, and biological properties, and the production of silver nanoparticles is present in many areas of interest of contemporary society, especially in biomedicine. Due to the low level of toxicity in human cells, silver nanoparticles are suitable candidates for use as antibacterial agents in dressings [6-19]. The involvement of silver nanostructures in numerous optimization directions of the biomedical field is one of the most attractive and impressive applications attributed to silver.

Tissue engineering based on wound healing requires the use of natural polymers, as the risk in terms of biocompatibility decreases significantly. Hydrogels are semi-solid systems formed by combining one or more hydrophilic polymers. They are among the most used dressings in treating wounds and burns. Hydrogels are designed to retain moisture at the application site while allowing oxygen to reach the area. These dressings offer several benefits, including biocompatibility, high treatment effectiveness, and user-friendly application. [20-27]. Alginate is a natural anionic polymer obtained from brown seaweed and is extensively researched and used for numerous biomedical applications due to its biocompatibility, low toxicity, relatively low cost, and easy gelation by adding

divalent cations such as Ca^{2+} [28-36]. Polyvinyl alcohol (PVA) is a non-crystalline synthetic polymer that consists mainly of the amorphous phase with a small amount of crystalline phase. PVA-based materials are of real interest in biomedical applications due to their biocompatibility, sensitivity to pH changes, availability, and relatively simple processing. Because its physical properties are similar to those of human tissues, it can be used in tissue engineering. Being a biocompatible polymer, PVA can adsorb protein molecules and allow cell adhesion without the presence of toxic effects [37-43]. Essential oils are widely used in the cosmetic, pharmaceutical, medicine, and food industries for their antibacterial and antifungal properties. They are a source of antioxidants and have an anti-inflammatory activity that promotes wound healing. Mandarin oil is an essential oil with a sedative effect; it can help reduce anxiety attacks, and it has analgesic properties as well as an anti-inflammatory effect (due to the Dlimonene component) and antimicrobial [44-47].

This paper was focused on obtaining hydrogel wounds with the main polymeric matrix composed of alginate and polyvinyl alcohol improved by the addition of silver nanoparticles and mandarin essential oil, known for their antimicrobial potential together with their influence for proper healing. In this sense, the materials were intended to be used as a potential solution for improving wound healing by offering the required properties and, more, by having an opposing activity with the microbial cells that may lead to infection.

This study research combines the antimicrobial properties of silver nanoparticles and mandarin oil within a hydrogel matrix, creating a unique dual-agent system that exploits the synergistic benefits of both components. Unlike conventional wound dressings, this innovative approach harnesses the collective antimicrobial strengths of silver and mandarin oil to promote enhanced wound healing. Mandarin oil is a treasure trove of antioxidants, which serve as potent neutralizers of free radicals. When paired with nanoparticles, these antioxidants can be stabilized and precisely delivered to specific skin sites, thereby promoting optimal skin health and potentially diminishing visible signs of aging. Developing a novel hydrogel comprising polyvinyl alcohol (PVA) and alginate (Alg) is particularly notable, as this unique blend of materials enables the creation of a hybrid with a carefully tuned set of properties. Specifically, this combination provides a harmonious balance of biocompatibility, and controlled release capabilities for active agents.

2. Materials and methods

The following materials were used to obtain silver nanoparticles: silver nitrate ($AgNO_3$), sodium hydroxide (NaOH), D-glucose ($C_6H_{12}O_6$), mandarin essential oil, and distilled water. For the synthesis of composite wound dressings,

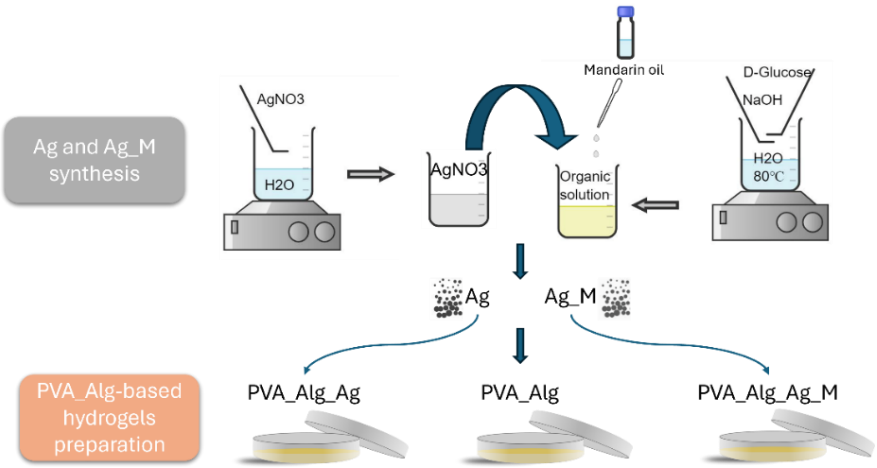
polyvinyl alcohol (PVA), sodium alginate (Alg), and distilled water were employed. All materials were purchased from Sigma-Aldrich.

The synthesis process of silver nanoparticles involves using a silver precursor solution, specifically silver nitrate, in this experiment, along with a reducing agent, which is D-glucose in this case. Metallic silver is produced through the reduction reaction. For this, a 0.01M solution of silver nitrate with a volume of 100 mL was prepared, along with an organic solution made by dissolving 1 g of D-glucose in a 1M NaOH solution at a temperature of 80^{o} Celsius. The next step is based on the precipitation process by adding the silver precursor solution drop by drop over the organic solution under continuous stirring, with the formation of silver nanoparticles being associated with the dark green color of the solutions in contact. The precipitate was thoroughly washed, followed by sequential centrifugations; finally, the drying process took place in the air for 72 hours. Regarding the synthesis process to obtain silver nanoparticles covered with mandarin oil, the same synthesis method was approached, but the presence of mandarin oil was fulfilled by adding a small amount to the silver-reducing solution.Preparation of the hydrogels starts by dissolving PVA and ALG individually in distilled water, forming two gel solutions with a 2% concentration for each one. A control sample was first prepared to achieve comparable results, being composed of both polymer solutions used in 1:1 volume ratio and coded as PVA_Alg. To obtain the second material, a small quantity of silver nanoparticles (Ag) was added to the control hydrogel and meticulously mixed for uniform distribution through the gel, referred further to PVA_Alg_Ag. The third hydrogel has the same polymeric composition and ratio, but in this case, the addition of silver nanoparticles was coated with mandarin essential oil and coded PVA_Alg_Ag_M in the paper. All three hydrogel formulations were subjected to a freezing process in the freezer for 24 hours, followed by a lyophilization process for 72 hours at -50 degrees. The samples were characterized for physical-chemical observations but also from the point of view of interaction with eukaryotic and prokaryotic cells in order to establish representative activities for the intended application. Table 1 presents the sample codes and their compositions, together with the schematic representation of hydrogel preparation.

Table 1.

Chemical composition and the codification of the dressings samples.

PVA (polyvinyl alcohol)	Alg (sodium alginate)	Ag (silver nanoparticles)	Ag_M (silver nanoparticles with mandarin oil)	Sample code
■	■			PVA_Alg
■	■	■		PVA_Alg_Ag
■	■		■	PVA_Alg_Ag_M



Scheme 1. Synthesis process for obtaining silver nanoparticles and hydrogels preparation.

X-ray Diffraction (XRD)

A diffractometer (PANalytical Empyrean model diffractometer purchased from PANalytical, Almelo, The Netherlands) was used to determine the crystallinity parameters of the silver nanoparticles. The X-ray diffraction equipment has a hybrid monochromator (2xGe 220) on the incident side and a parallel plate collimator mounted on the PIXcel 3D detector on the diffracted side. The measurements (Grazing Incidence X-ray Diffraction) were operated at room temperature with $\omega = 0.5$ ° angle of incidence for Bragg angle 2θ between 10 ° and 80 °. Also, the radiation Cu Kα has $\lambda = 1.5406$ Å (40 mA and 45 kV).

Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) Inspect F50 equipment purchased from Thermo Fisher - FEI (Eindhoven, The Netherlands) was used to investigate the morphological structure of the silver nanoparticles and further of the alginate-based hydrogels. A carbon-bearing slide was applied to the stab, followed by the silver nanoparticle powder, and inserted in the microscope's analysis chamber. The same procedure was also used for hydrogels, which were covered with a thin gold film for 20 seconds. The micrographs were performed in secondary electron beam and electron beam scattering with an energy of 30 keV.

Transmission Electron Microscopy (TEM)

For preparing the nanoparticles to analyze them with a high-resolution 80200 Titan Themis transmission electron (FEI, Oregon, USA), the powder was dispersed in water by ultrasonic treatment for 15 minutes and placed on a copper-coated grid. This microscope achieves images in transmission mode at a voltage of 200 kV, with point and line resolution of 2 Å and 1 Å, respectively.

Fourier transform infrared spectroscopy (FTIR)

FTIR spectra were achieved using a ZnSe crystal of Thermo iN10-MX FTIR spectrometer (Thermo Fisher Scientific, Waltham, USA.). The measurements were performed between 4000 – 400 cm^{-1}, and for every spectrum, 32 scans were made. The spectra results were converted in absorbance using the software Omnic Picta (Thermo Scientific).

Biological evaluation

Biocompatibility testing of materials at the level of cell cultures was performed on dermal fibroblasts CCD1070Sk (MEM medium – Minimum Essential Media with 2 mM L-glutamine). Based on this quantitative colorimetric method, it is possible to assess cell proliferation. The method is based on the reduction of a yellow tetrazolium salt MTT (3-(4,5dimethylthiazolium)-2,5diphenyltetrazolium bromide) to dark blue formazan. The optical density (OD) of the solubilized formazan is evaluated spectrophotometrically, obtaining an absorbance-dye concentration-number of metabolically active cells in the culture function.

A modified version of the disc diffusion method was utilized to assess the obtained dressings' antibacterial effectiveness. Each strain (*Escherichia coli* and *Staphylococcus aureus*) was used to produce microbial suspensions that were then diluted to have an optical density of 0.5 McFarland (1.5 108 CFU (colony forming units)/mL) in sterile saline buffer. The whole surface of the nutrient agar Petri dishes was inoculated using these microbial suspensions. Following inoculation, samples of sterile dressings measuring 5 mm in diameter were aseptically placed on the inoculated agar surface. The diameter of the growth inhibition zone (mm) was measured following incubation (24 hours at 37 °C). A larger inhibition zone shows that the dressing has a stronger antibacterial action.

3. Results and discussion

Silver nanoparticles (Ag) and mandarin oil silver nanoparticles (Ag_M) characterization

Fig. 1 shows the diffractogram for Ag – silver nanoparticles and silver nanoparticles coated with mandarin essential oil – Ag_M.

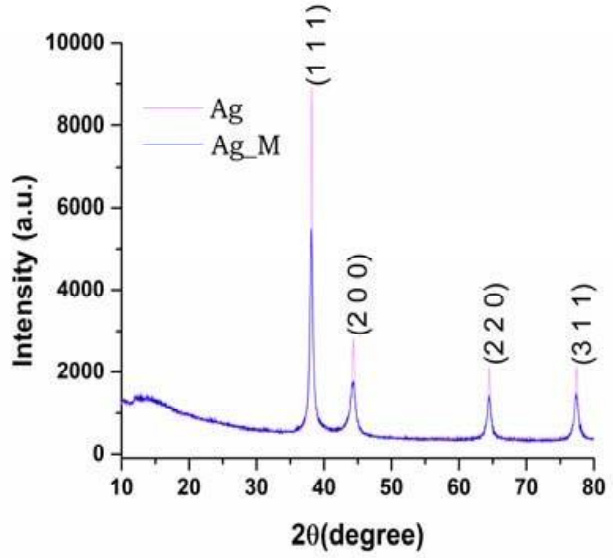


Fig. 1. XRD diffraction result for Ag and Ag_M nanoparticles.

The only phase identified in both samples is silver. The samples show diffraction interference specific to planes with Miller indices (111), (200), (220), (311), in accordance with the ICDD sheet PDF4+ 00-004-0783 for silver [48,49]. The high degree of crystallinity of the sample is highlighted by the intensity of the diffraction lines; thus, narrow, well-defined peaks can be observed. The only difference between the analyzed samples is still related to their degree of crystallinity; in the case of Ag_M being identified, a decrease in the intensity of the peaks, a fact due to the presence of mandarin essential oil in the analyzed sample. The average crystallite size was calculated with the Debye-Scherrer formula as follows:

$$D = \frac{0.9\,\lambda}{\beta\cos\theta}$$

where "D" (average crystallite size) is calculated by mathematically involving the characteristic wavelength of the equipment represented as "λ" with a value of 0.1540 nm, by the full width at half maximum (FWHM) represented as “β” and by the diffraction angle represented as “θ” [50,51].

Table 2 contains information regarding the average crystallite size of the samples, where the influence of mandarin essential oil is observed. Smaller values of the crystallite size are identified in the case of Ag_M, compared to Ag.

Table 2.

Calculated average crystallite size for Ag and Ag_M.

Average crystallite size	16.52 ± 2.79 nm	Ag
	10.09 ± 0.47 nm	Ag_M

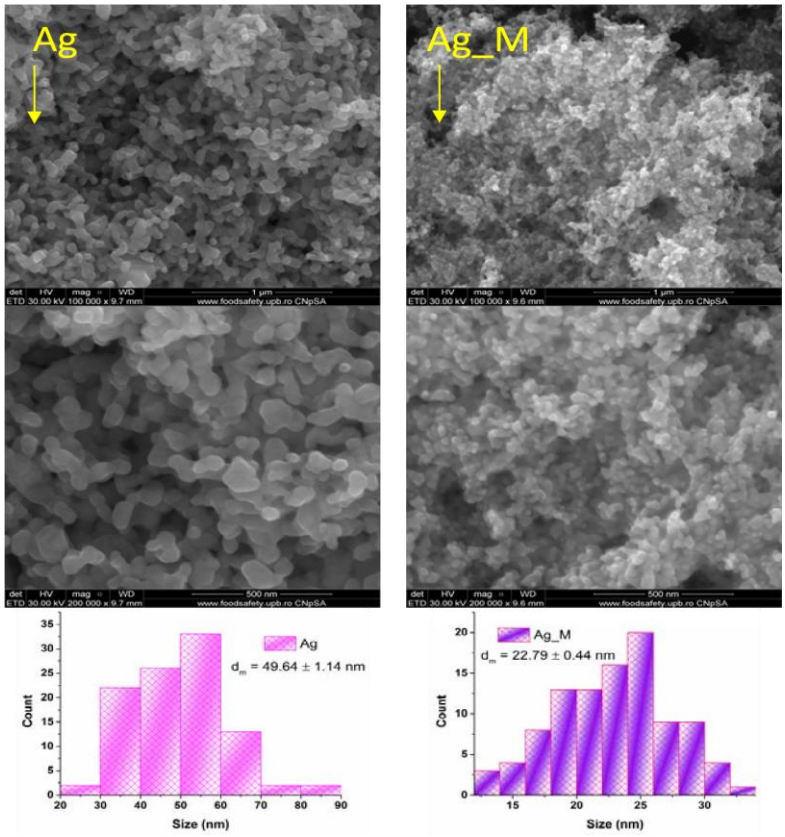


Fig. 2. SEM micrographs and size distribution for Ag and Ag_M nanoparticles.

After identifying silver as a single phase in Ag and Ag_M samples, the next step involves the evaluation of the morphology and dimensions of the nanoparticles. In this sense, Fig. 2 illustrates the SEM micrographs at two magnifications (x100 000 and x200 000) and the measured size of the nanoparticles represented as a size distribution histogram.

The morphological characteristics of the silver nanoparticles samples show similarity from the degree of agglomeration point of view, numerous organized nanoparticles together, with a high agglomeration tendency.

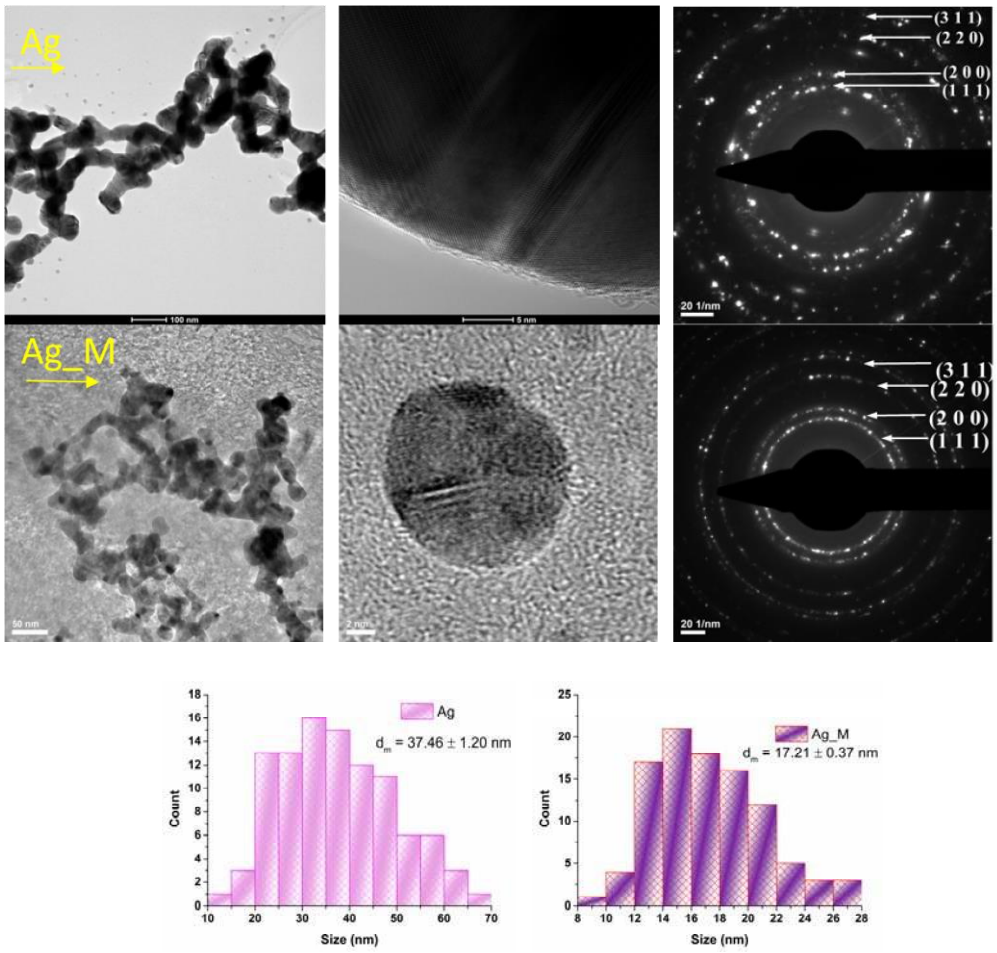


Fig. 3. TEM micrographs, SAED pattern and size distribution for Ag and Ag_M nanoparticles.

This agglomeration tendency is due to the nanometric size of the particles, the calculated average size being ~49 nm in the case of Ag and ~22 nm in the case of Ag_M, respectively. Notably, the presence of mandarin essential oil led to a significant reduction in the average crystallite size, and a similar trend was observed for the particle size, where the value of Ag_M was nearly halved. The shape of the nanoparticles in the case of the Ag sample is quasi-spherical, and with a tendency to elongate, while in the case of the Ag_M sample, well-defined spherical shapes are observed; this result also highlights the impact of mandarin oil on the physicochemical properties.

The TEM microscopy analysis was used to observe the morphological and dimensional particularity of the nanoparticles and the crystallinity represented by the SAED pattern at a better resolution.In Fig. 3, the influence of mandarin oil on the properties of silver nanoparticles is also confirmed. In the case of the Ag_M

sample, the spherical morphology of the particles is maintained, compared to the Ag sample, where quasi-spherical shapes of the nanoparticles are identified. The dimensional characteristics resulting from TEM, show halved values for the Ag_M (~17 nm) sample compared to the Ag (~37) sample, which is observed in the size distribution of the nanoparticles. The rings formed by the bright spots represented in the SAED pattern for both silver samples confirm the only phase identified, namely silver, by the presence of Miller indices, a result also correlated with the XRD analysis.

Wound dressings characterization – PVA_Alg, PVA_Alg_Ag, and PVA_Alg_Ag_M.

The composite hydrogels were analyzed using FTIR analysis to identify the functional groups observed at a specific wave number. Fig. 4 shows the infrared spectra for PVA_Alg, PVA_Alg_Ag, and PVA_Alg_Ag_M, respectively.

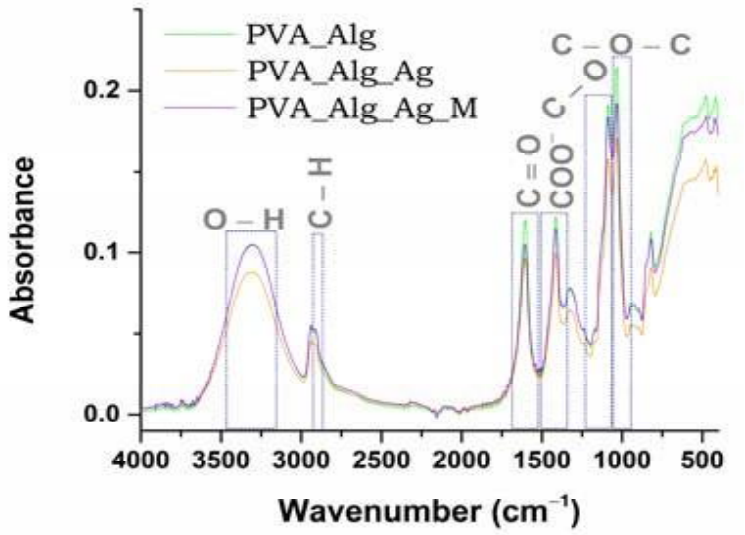


Fig. 4. FTIR spectra recorded for PVA_Alg, PVA_Alg_Ag, and PVA_Alg_Ag_M wound dressings.

All the spectra obtained show the wave number from approximately 3200 cm^{-1} characteristics of the O – H functional group, indicating the hydrophilic feature of the samples. The vibrational bands in the range 1500-1600 cm^{-1} demonstrate the presence of the C = O chemical group found in the structure of polyvinyl alcohol. 1409 cm^{-1} is the wavenumber characteristic for COO^- carboxyl group, particularly for alginate. At the wave number of approximately 1030 cm^{-1}, the C – O – C vibration specific to alginate is present, and at the same time, the C – O stretching specific to polyvinyl alcohol is identified at 1088 cm^{-1}. 2900 cm^{-}

[1] represents the wave number identified for the C – H group in all polymeric structures.

SEM electron microscopy analysis was used to observe the morphology of the dressings as well as to evaluate the uniform distribution of silver nanoparticles within their composition. In this sense, all three dressings were observed microscopically at magnifications of x100 and x500, respectively, under the same conditions so that there could be comparative differences between them.

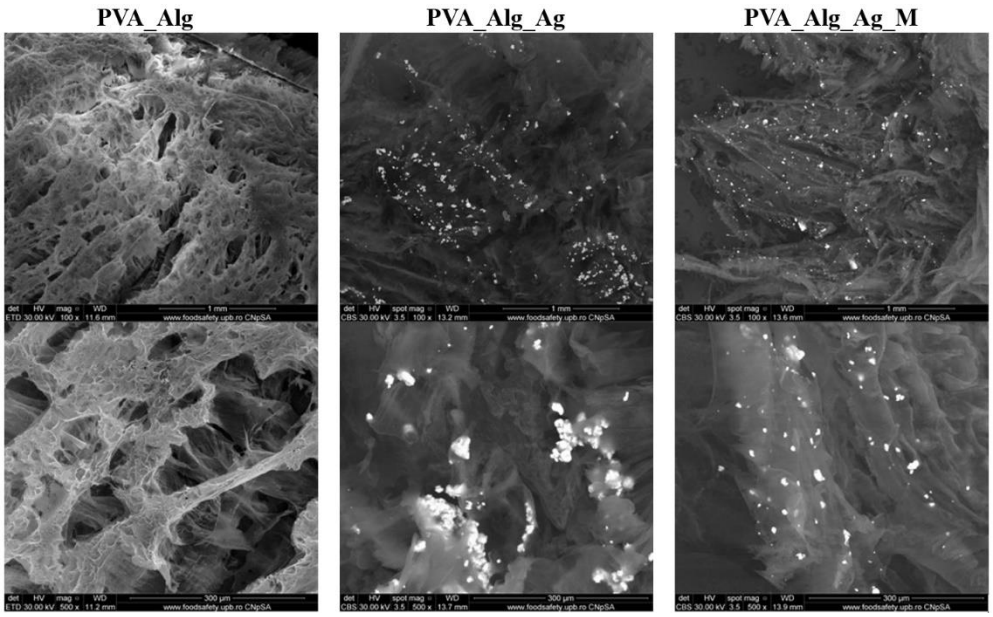


Fig. 5. SEM micrographs for PVA_Alg, PVA_Alg_Ag, and PVA_Alg_Ag_M wound dressings.

Fig. 5 highlights important morphological details related to a dressing found in the case of the three composite materials obtained, with applications in the treatment of wounds. The first important property that PVA_Alg, PVA_Alg_Ag, and PVA_Alg_Ag_M dressings fulfill is the porous structure. This type of structure is essential when the proper healing of a wound is desired, as it is possible to maintain an optimal level of oxygen as well as cellular proliferation. The incorporation process of silver nanoparticles was successful; they were observed in the PVA_Alg_Ag and PVA_Alg_Ag_M samples, both on the surface and in the polymeric structure of the dressings, with optimal homogeneity distribution. The dressing containing silver nanoparticles coated with mandarin oil (PVA_Alg_Ag_M) shows smaller particle agglomerations, and their distribution is improved; the essential oil also has a beneficial influence on the physicochemical properties in this case.

Considering the application of these composite dressings, it is necessary to evaluate them in contact with skin cells to observe their interaction, especially regarding cell proliferation.

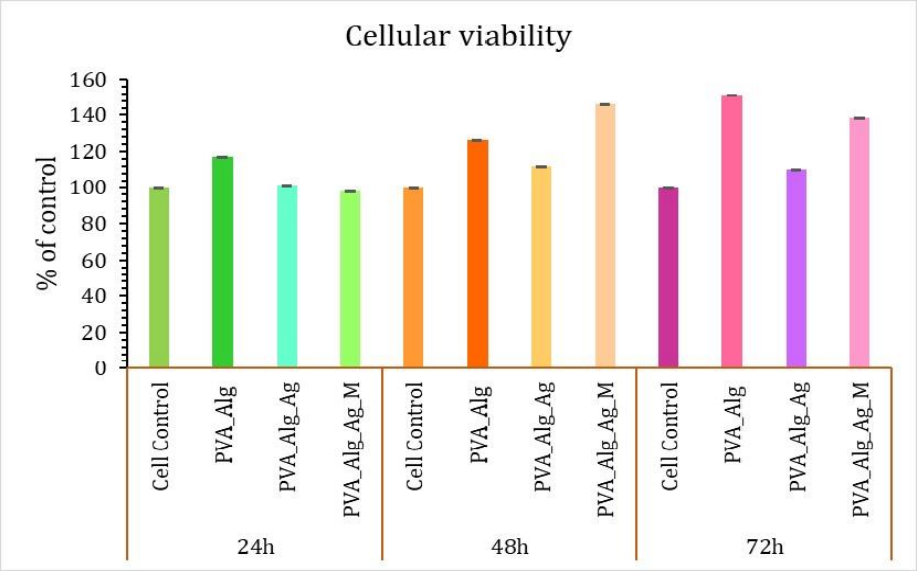


Fig. 6. Biological evaluation in terms of cellular viability for PVA_Alg, PVA_Alg_Ag, and PVA_Alg_Ag_M wound dressings on dermal fibroblasts CCD1070Sk after 24, 48, and 72 hours.

The three synthesized composite dressings were cultivated in contact with dermal fibroblast cells, their interaction being evaluated after 24, 48, and 72 hours, respectively. These evaluations were reported to the control cells in order to observe the influence of the tested dressings, and the results are graphically represented in Fig. 6. The first and most important result refers to the fact that all the samples have equal or higher values compared to the control cells, which was observed after all three periods of incubation and testing. After 24 hours, the sample that has the highest percentage of viability in relation to the control is PVA_Alg, and this is very important because the polymer base represents the largest part of the obtained dressings, and thus, it is demonstrated that even from the first contact it shows biocompatibility, and it even supports cell proliferation. After 48 hours, the PVA_Alg_Ag_M dressing represents the sample with the highest viability percentage, highlighting the involvement of silver nanoparticles coated with mandarin essential oil. Considering that silver nanoparticles with mandarin oil are present in the polymer structure, a longer time than 24 hours is needed to deliver outside the matrix. However, after 72 hours of incubation, the test results show that all three dressings stimulated cell proliferation, with PVA_Alg_Ag_M exhibiting the most pronounced effect. The main activity of

silver nanoparticles is recognized as an ideal solution for combating bacterial infections. At the same time, mandarin essential oil exhibits similar antimicrobial properties and is involved in skin regeneration. The anti-infective activity of the dressings was tested using the growth inhibition zone method on two bacterial strains *E. coli* and *S. aureus*, known as pathogens often found in wound infections. Fig. 7 graphically represents the result where the control is represented by ampicillin, an antibiotic recognized for its activity.

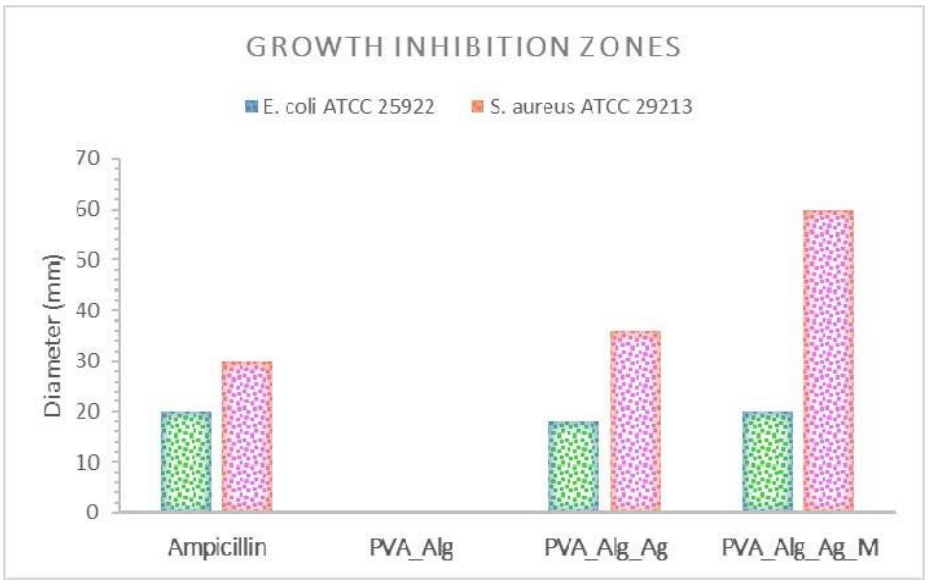


Fig. 7. Biological evaluation in terms of antimicrobial activity for PVA_Alg, PVA_Alg_Ag, and PVA_Alg_Ag_M wound dressings on *Escherichia coli* and *Staphylococcus aureus*.

The result of the growth inhibition zone test demonstrates that, in general, ampicillin as well as PVA_Alg_Ag and PVA_Alg_Ag_M dressings have an increased effect against the strain of *S.aureus* but also against *E. coli*. What was expected and can be found in the result is the fact that the PVA_Alg sample does not show activity against the two bacterial strains tested. Another thing worth noting is that the PVA_Alg_Ag_M sample presents the best result on both bacterial strains, with an emphasis on *S.aureus*, being also demonstrated in the result of this test, the activity of mandarin oil, which produces only beneficial effects for the treatment of wounds.

4. Conclusions

This study is based on the development of dressings with applications in the healing and eradication of wound infections, starting from synthesizing silver nanoparticles that were afterward coated with mandarin oil and homogenized in a biocompatible polymer matrix. The sample of interest was throughout the study PVA_Alg_Ag_M because it represents the composite that offers all the individual properties of the materials involved in one place. The discussion and interpretation of the results were made in comparison with the other two dressings (PVA_Alg and PVA_Alg_Ag), highlighting the potential that PVA_Alg_Ag_M has in the targeted application. Physicochemical analysis of Ag and Ag_M samples revealed that the presence of mandarin essential oil had a significant impact from the outset, as evident in the decreased crystallite and particle size, as well as alterations in particle morphology, which contributed to the formation of well-defined spheres.In the case of the biological evaluation, the best results were obtained in the case of the PVA_Alg_Ag_M sample both in terms of cell proliferation observed even after 72 hours and in terms of antimicrobial activity on a Gram-positive and a Gram-negative strain.

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