ZnO@ESENTIAL OILS BASED SODIUM ALGINATE/SILK FI-BRO-IN/HIALURONIC ACID SCAFFOLDS FOR WOUND APPLICATIONS

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Abstract. *Interest to finding a solution to help reduce the cases of microbial infections in wounds is very high. The concern is all the greater as antibiotic resistance has become an increasingly common problem. In order to find a new way to synthesize a wound dressing which has superior prop-er-ties, sodium alginate, hyaluronic acid and silk fibroin functionalized with zinc oxide and clove and oregano essential oils were used. The results are encouraging for using this kind of wound dressing as an efficient treatment, showing antibacterial activity against wound patho-gen Staph-ylococcus aureus.*

Keywords: nanocomposite; antibiotic resistance; zinc oxide; wound dressing, tis-sue regeneration
DOI https://doi.org/10.56082/annalsarsciphyschem.2023.2.28

1. Introduction

Microbial infections are a common medical problem assigned to various internal (prostheses, percutaneous implants) and external (wound dressings, urinary catheters, etc.) biomedical devices, especially those that come in direct contact with affected skin or mucous membranes. Such an infection is especially important to be taken into consideration for permanent medical devices, which should be used for the rest of the patient's life. These medical devices are usually orthopedic implants that replace parts of the bone sys-tem, arterial grafts, stents, and other components that support vital functions [1].

When a post-surgical infection occurs and it cannot be resolved by classical medication, the infected internal device must be removed and replaced [2][3][4].

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Nowadays, medication methods are based on the administration of antibiotics for a long period of time [4]. Usually, the long term medication leads to complications due to their organ toxicity, low specificity, low efficacy and bacterial resistance [5] [6] [7]. The best way to avoid this medical problem is to develop an effective and also preventive measure based on an antibacterial system which is capable of providing antibiotics, and other antimicrobials (i.e. antimicrobial peptides, natural extracts, essential oils), etc. in situ, at the site of the wound [6][8][9]. To overcome the antibiotic resistant infections, the medical solution is to promote the administration of a lower dose of bactericides directly to the infected area or, even better, the de-livery of nanoparticles with an antimicrobial activity [9].The use of active substances as essential oils and nanoparticles in the composition of antibacterial systems, has a number of advantages, e.g. high efficacy at low doses, simultaneous ad-ministration of single or multiple drugs, a stable level of concentration of the drug and a lower occurrence of side effects [10].

Recently, a general trend was to develop biocompatible, bioresorbable and easy to be produced materials, such as Ag, ZnO, MgO, Cu nanoparticles embedded into hydrogels. Hydrogels are ideal biomedical materials, being obtained from almost every hydrophilic polymer, with controlled porosity, density and absorbability [10]. By definition, hydrogels are a 3D polymer network capable of absorbing large amounts of liquids without changing their structure [10]. Hydrogels can be used to cover catheters, lenses, stents, bone im-plants, as well as to heal wounds and inject locally the drugs [11]. Despite the large num-ber of scientific studies based on wound dressing materials, there is no evi-dence of the use of porous matrices formed by silk fibroin / hyaluronic acid / sodium alginate and its functionalization with ZnO nanoparticles and essential oils [12].

The materials used in the design of tissue engineering matrices (natural polymers, synthetic polymers or hybrid materials) must incorporate the following properties: (i) excellent bioactivity and biocompatibility; (ii) mechanical properties similar to the tissue (ii) the supporting role, (iii) to produce an optimal environment for the adhesion and proliferation of the cells involved in the regeneration process [13][14].

Natural polymers such as fibroin, sodium alginate and hyaluronic acid, are preferred choices for the synthesis of biocompatible matrices. The major advantage of the use of the natural polymers compared to the synthetic ones is their biocompatibility. The natural polymers are recognized by the physiological environment, being degraded by processes of metabolic degradation without the risk of releasing toxic compounds. Silk fibroin is a fibrous protein consisting of a chain of light (L) and heavy (H) polypeptides linked by a disulfide bridge forming an H-L complex. The heavy (H) polypeptide chain is the major constituent in the protein structure that gives the material mechanical strength. The poly-peptide chain (L) is more hydrophilic and relatively elastic, so it does not contribute to the consolidation of mechanical strength [15].

Sodium alginate is one of the most studied natural polymers due to its unique properties, Sodium alginate is a linear polysaccharide consisting of two units of D-manuronic acid and one unit of L-guluronic acid [16]. The properties that have made this polymer remarkable in the field of biomedical research are: biocompatibility, hydrophilicity and biodegradability. For these characteristics, is the most used polymer in the design and synthesis of materials which form implantable medical devices without potentially toxic or immunogenic actions [17].

Hyaluronic acid is a polysaccharide that contains alternating units of N-acetyl-D-glucusamine and glucuronic acid. The polymer is a part from human tissues, predominantly found in soft tissues, which gives them elasticity, flexibility and optimal mechanical strength. The polymer is also involved in the healing process of skin or mu-cosal injury. Hyaluronic acid promotes the formation of the extracellular matrix (ECM) through the chemical interactions. The properties of hyaluronic acid depend on its molecular weight [18].

Among various types of nanomaterials which have been developed, nanostructured metal oxides (NMOs) have recently attracted a great attention. The nanostructured oxides of the materials, such as: Zn, Fe, Cu, Au, Ag showed interesting nanomorphological, bio-compatible, non-toxic and catalytic properties [18]. These materials have an important role in improving electron transfer kinetics and a strong absorption capacity, due to their semiconductor nature [19]. For this interesting properties, they can be included in a wide range of applications, from drug-controlled release systems to biosensors [20].

The antibacterial properties of ZnO have been extensively studied to control pathogens in less aggressive ways than conventional antibiotics or antivirals. ZnO has been shown to be effective against the most responsible pathogens such as Escherichia coli [21][22], S aureus [22] [23] and Klebsiella pneumonia [24]. The mechanism by which ZnO acts as a barrier to reduce the spread of microorganisms in the physiological environment is explained as follows: the semiconductor character of ZnO attributed to the band gap value of 3.28V favors the formation of metal ions, and by photo regeneration processes reactive oxygen species (superoxide (O2-) and hydrogen peroxide (H2O2)) are formed

[22][25][24][26]. The reactive oxygen species cause damage to the cellular components: nucleic ac-ids, proteins, enzymes, cell membranes. Remarkable antibacterial properties of ZnO nanoparticles depend on the shape, morphology, and its specific surface area [25][24][26].

A different approach to design innovative materials with antibacterial properties is the use of clove or oregano essential oils. Oregano essential oil is known to be the most effective natural antimicrobial followed by clove essential oil [27].

The main antibacterial components in oregano oil are carvacrol and thymol. Both compounds have hydrophobicity which allows them to attach to the cell membrane. They cause membrane expansion, increase its permeability, interact with cellular proteins, in-hibit cellular respiration and affect the process ion transport. The compounds have an an-tioxidant character that inhibits the peroxidation of liposomal phospholipids directly de-pendent to the concentration [27].

Clove essential oil contains eugenol (hydroxyphenyl propene), which belongs to the class of aromatic phenols [28]. The compound is highly volatile, with low chemical stability. The mechanism by which eugenol interacts with pathogen agents and causes their death follows: (i) cause cell membrane damage made from fatty acids, (ii) produce severe changes in cell morphology, (iii) affect the transport of ions and ATP, (iv) induce ROS formation and (v) inhibit the activity of bacterial enzymes [28][29].

The paper aims to design and to manufacture a wound dressing containing silk fibroin, sodium alginate and hyaluronic acid that will be functionalized with ZnO and essential oils, with applications in tissue engineering. The obtained material is designed to be used as an antimicrobial wound dressing, which could be used for any type of wounds, including burns [30].

2. Materials and Methods

2.1. Materials

For the synthesis of the composite fibroin-sodium alginate-hyaluronic acid the following materials were used: sodium alginate powder (purity 99.98%, Sigma-Aldrich), hyaluronic acid (Sigma-Aldrich), silk fibroin solution concentration 50mg / mL (20mL, Sigma Aldrich). For the functionalization of the composite were used: zinc oxide and essential oils. For the synthesis of zinc oxide were used: zinc acetate dihydrate (purity 98%, Sigma-Aldrich), sodium hydroxide (purity 98%, Sigma-Aldrich), absolute ethyl alcohol. The essential oils used were oregano essential oil (DoTerra), clove essential oil (DoTerra).

2.2. ZnO nanopowders syhthesis

ZnO nanopowders were obtained by using modified poliol method. 5 grams of Zinc acetate dihydrate ($Zn(CH_3COO)_2 \cdot 2H_2O$) was inserted into a round bottom baker. Ethylene glycol (HOCH2CH2OH) was added as a liquid media for the synthesis of zinc oxide, the baker was mounted onto a distillery system at 120°C for 12 hours. After the 12 hours, the obtained white precipitate was washed several times using a centrifuge at 15.000 rpm for 15 minutes. The obtained powder was used as is, no further heat treatment was applied.

Afterwards, the obtained ZnO nanopowder was coated using 1% solution of pure essential oils of oregano and cloves using the procedure as follows: 1% of oil, calculated for the mass of ZnO used, was diluted into ethanol. The powder was placed into the solution and left to stir until the ethanol had evaporated. All the procedure was made at room temperature.

The dressings were obtained starting from a solution of 4 % sodium alginate over which was added 0.016 g hyaluronic acid and silk fibroin. In order to obtain the final composites, the in samples which contain solution of sodium alginate / hyaluronic acid / silk fibroin were added zinc oxide nanopowders coated with essential oils in proportions of 1, 3 and 5%, respectively. The samples thus obtained were then frozen and lyophilised. After the lyophilisation process, the samples were immersed in 3% calcium chloride solution in order to crosslink the sodium alginate. After immersion process, the samples were left to dry in the air.

2.3. Characterization methods

The obtained samples were characterized in terms of mineralogical composition, morphology, antimicrobial activity using the method of minimum inhibitory concentration (MIC) and the Biofilm method. X-ray diffraction analysis was performed using a PANalytical Empyrean diffractometer in Bragg - Brentano geometry equipped with a Cu - anode X- ray tube and PIXcel3D detector. The analysis was acquired on the $2\theta = 20 – 80°$ angle range.

The morphology of the obtained nanopowders and dressings was studied using a scanning electron microscope QUANTA INSPECT F equipped with an energy dispersive spectrometer (EDAX).

3. Results

3.1. X-ray and SEM analysis on ZnO Nanopaticles

The as obtained nanoparticles were characterised in what concerns their structure and morphology. The obtained results are presented in Figure 1.

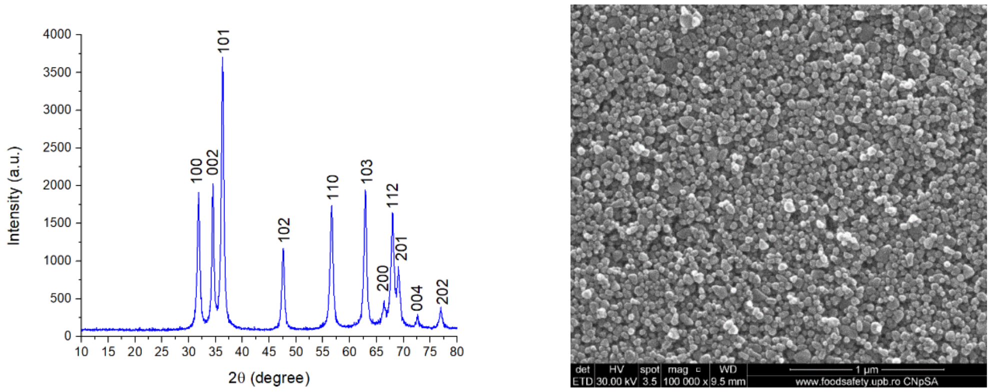
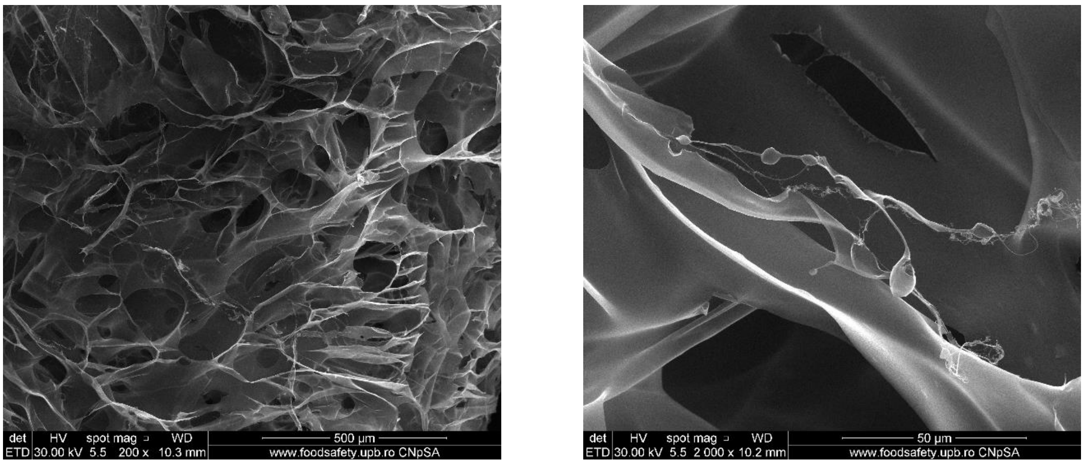


Fig 1. X-ray diffraction pattern of ZnO nanopowders (left) and SEM image (right).

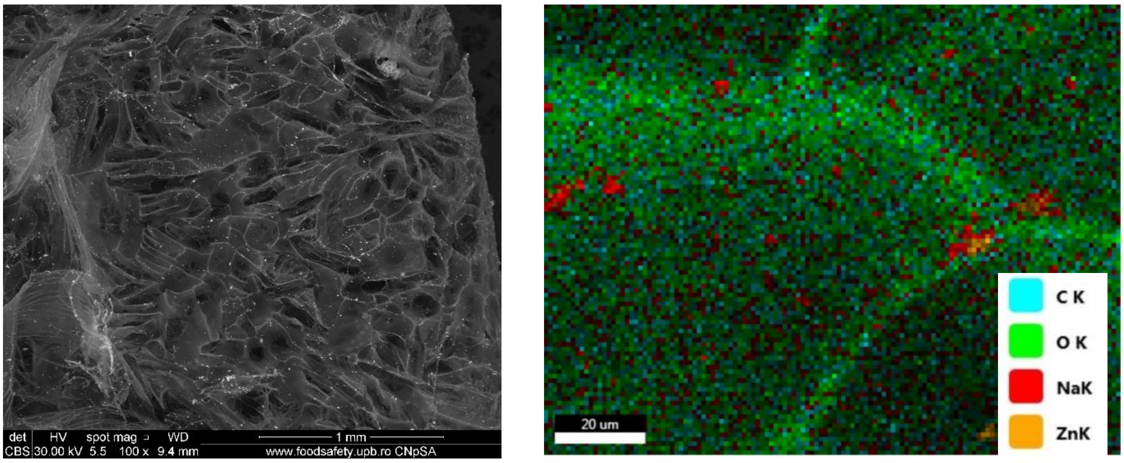
The XRD spectra of synthesized ZnO nanopowders revealed only a pure and crystalline phase that match with the pattern of a hexagonal structure according to ICDD no. 01-080-4199. The crystallite size calculated form the pattern reveals a nanopowder with an average of 13.26 nm. From the SEM images, we can see that the ZnO sample revealed nanostructured material with spherical and polyhedral morphologies. The particle size of ZnO nanoparticles are in the range of 15 to 35 nm. This being said, the zinc oxide nanoparticles are mono an polycrystalline.

3.2. Scanning electron microscopy (SEM) images for the obtained scaffolds

Following the synthesis and coating the nanoparticles with the essential oil, the morphology of the obtained scaffolds is presented in figure 2 for all samples, with and without zinc oxide nanoparticles.



a)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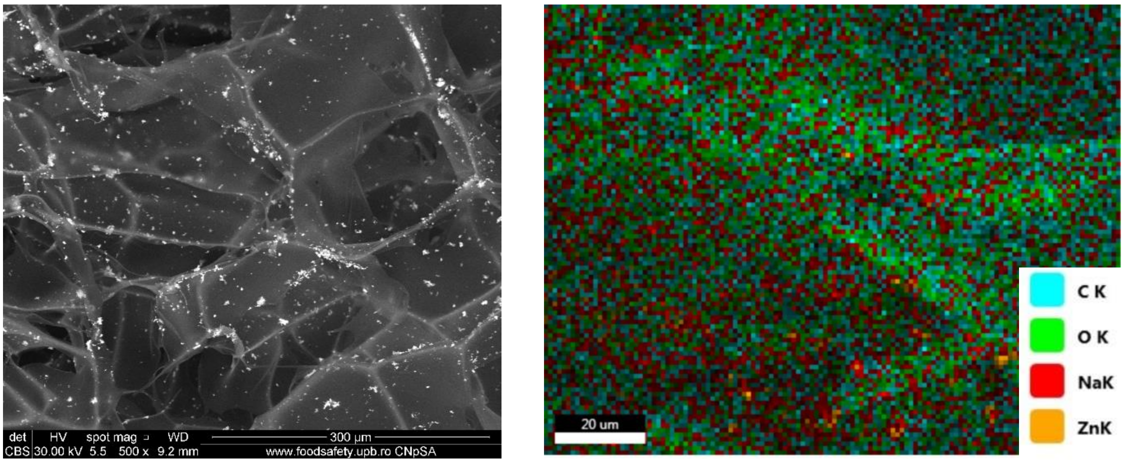
b)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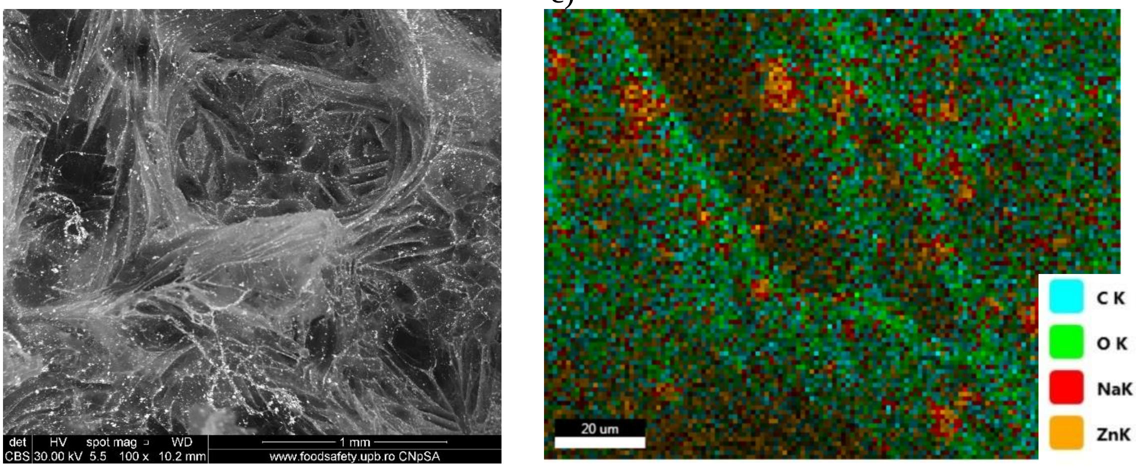
c)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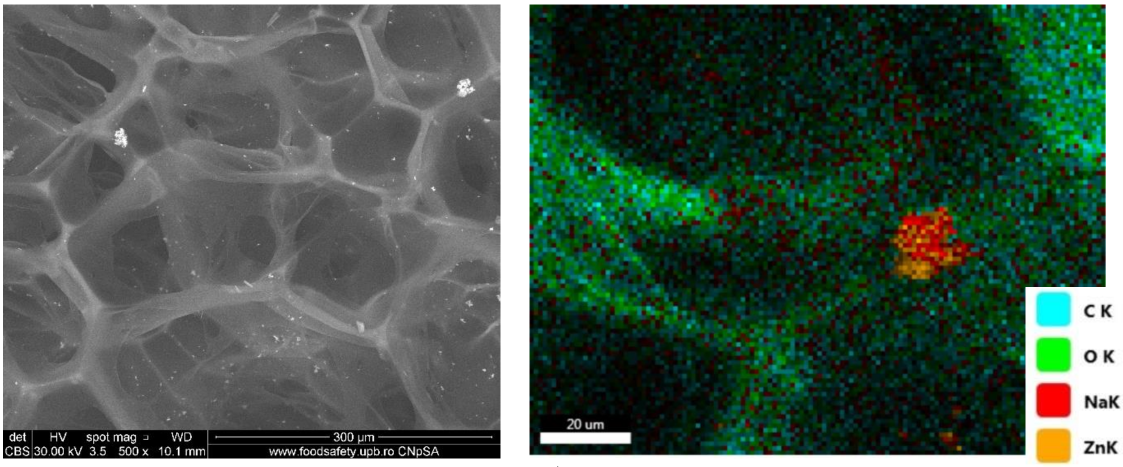
d)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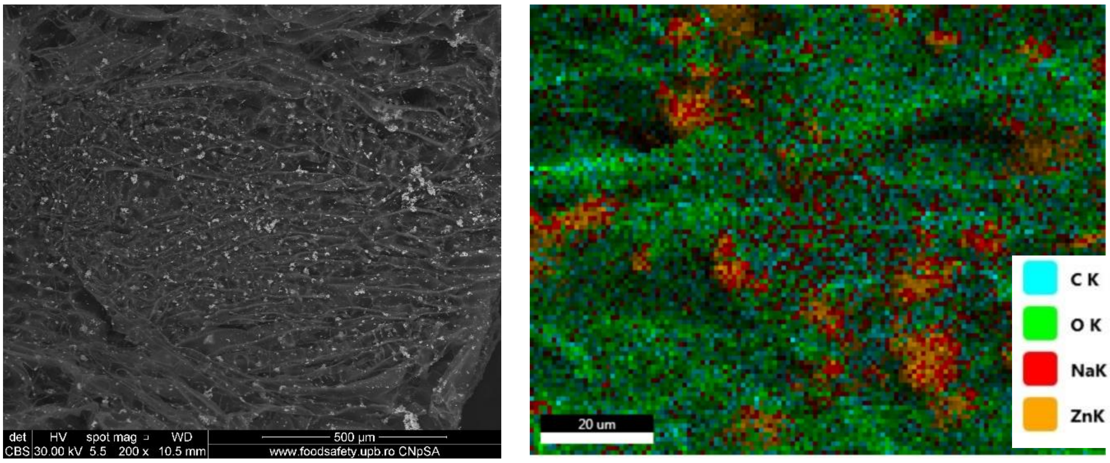
e)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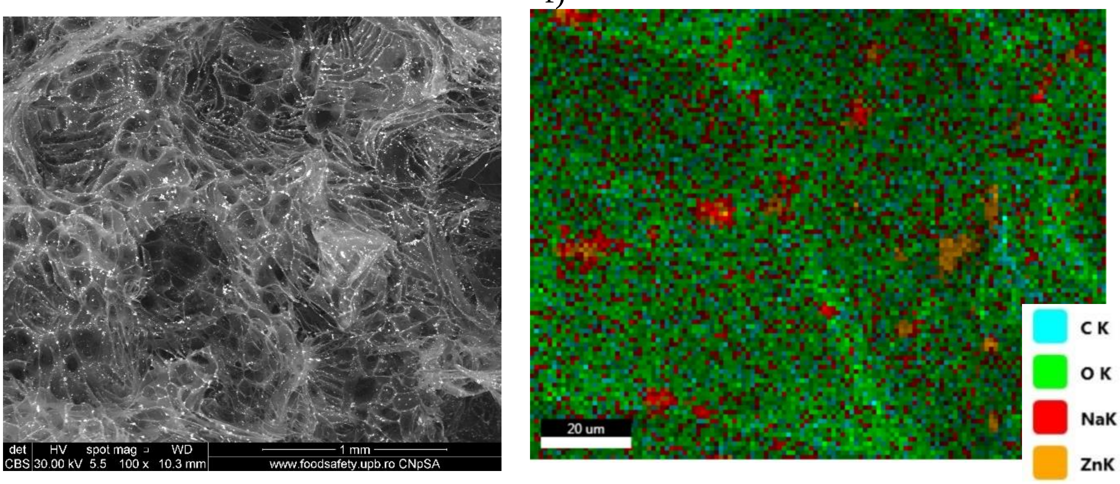
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NaK
ZnK

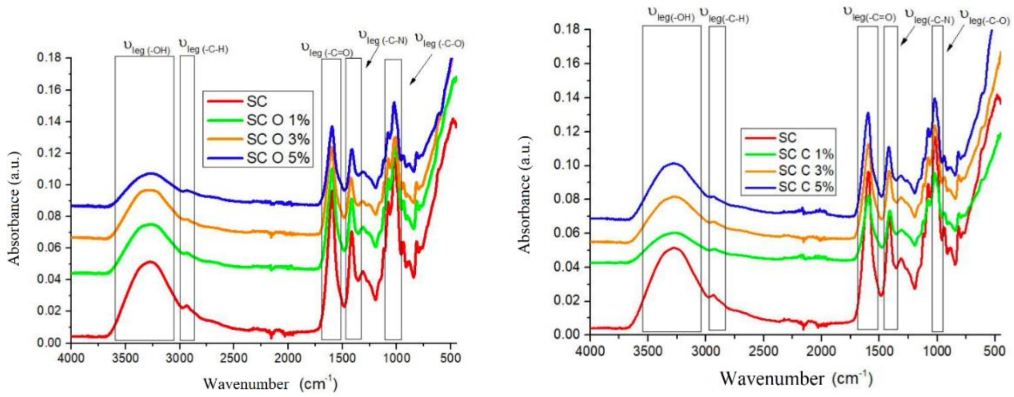
g)

Fig 2. Scaffolds without coated ZnO a), with 1% essential oil (cloves) b), with 3% essential oil (cloves) c) and with 5 % essential oil (cloves) d), with 1% essential oil (oregano) e), with 3% essential oil (oregano) f) and with 5% essential oil (oregano) g)

The SEM images obtained for the dressings with zinc oxide nanostructures as well as the one without ZnO reveal a very porous morphology, typical for lyophilized samples, with a very uniform distribution of zinc oxide for all three concentrations. This morphology is also confirmed in the elemental distribution images. In the detailed image obtained for the dressing without zinc oxide, the silk fibroin can also be seen. Also, in the case of of using zinc oxide coated with oregano oil, the distribution of zinc oxide as well as the morphology of the samples is similar with the ones that uses clove as the essential oil. This means that the oil has no influence on the morphology and distribution of the zinc oxide in the samples.

3.3. Fourier transformated infrared spectometry

In Figure 3 shows the FTIR spectra for oregano oil composite material (a) and clove oil composite material (b) is presented.



a) b)

Fig. 3. FTIR spectra for oregano oil a) and clove oil b) composite material

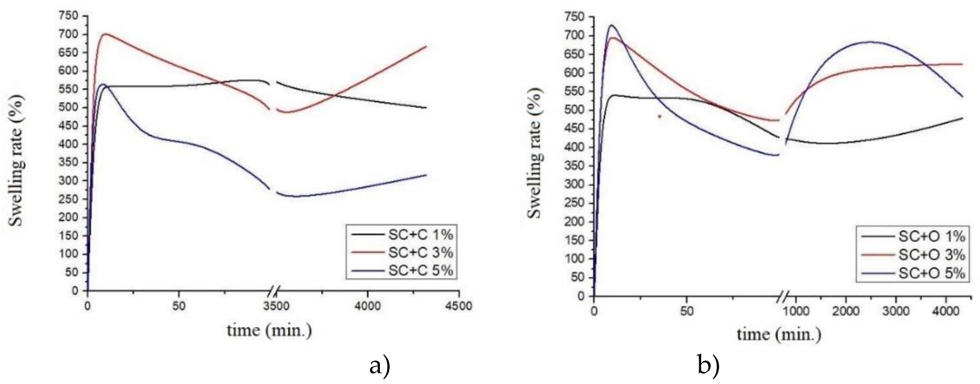
Absorption spectrum of the analysed samples shows absorption bands with the maximum absorption at 3279 cm^{-1} the vibration of the bond between the oxygen atom and the hydrogen atom of the hydroxyl group. The absorption band may also indicate the presence of secondary -NH- groups in the structure of fibroin and hyaluronic acid. The absorption band with the absorption maximum at 2929 cm^{-1} indicates the absorption of the bond between the carbon atoms in the chain and the hydrogen atoms. The absorption band with the maximum absorption at 1600 cm^{-1} is characteristic for the vibration of the carbonyl bond. The absorption band with the maximum absorption at $1424cm^{-1}$ indicates the presence of -C-N bonds once again confirming the presence of amino groups, as well as the absorption band with the maximum absorption at 1018 cm-1 which confirms the presence of hydroxide groups.

The difference between the presented spectra is the essential oil used, respectively in figure 3 a) samples containing oregano essential oil were analysed and in figure b) clove essential oil. Compared between the samples with oregano and clove oil, it is observed that the absorption band characteristic for the hydroxyl group changes its curvature, in the case of oregano oil, as the concentration increases the band becomes less sharp, compared to the samples with clove oil where with the increase in band concentration becomes sharper.

3.4. Swelling test

An initially determined volume was immersed in distilled water to determine the mass of water incorporated in the sample and the swelling rate. A 24-well plate was used for analysis of the samples. The results are shown in Figures 4 a) and b)

A more pronounced degradation is observed for samples containing a higher concentration of zinc oxide so that the higher the concentration of zinc oxide, the more pronounced and faster the degradation will be. All samples kept their original shape and structure after 72h, there is no dissolution, the samples are stable in water.



a) b)

Fig. 4. swelling rate, a) clove oil and b oregano oil composites

For the sample containing 1% ZnO + clove oil, a marked swelling is observed in the first hours after immersion, it has a high stability in water, after about 2 days the sample begins to degrade with a very low degradation rate. The sample containing 3% ZnO + clove oil swells with a higher swelling rate compared to the sample containing 1% ZnO + clove oil, so the swelling rate and degradation rate are more pronounced. For the sample containing 5% ZnO + clove oil, the highest rate of swelling and degradation is observed compared to the other samples.

For functionalized samples with oregano oil, an inflation rate is also observed, which increases with increasing inflation rate, as well as the degradation rate, which is more pronounced than the samples with clove oil.

3.5. Antimicrobial activity

The antimicrobial activity of the obtained nanopowders and dressings were tested in an opportunistic Gram positive wound relevant pathogen, namely Staphylococcus aureus ATCC 25923 strain, which was maintained on nutrient broth supplemented with 20% glycerol at -80 ° C. For the antimicrobial tests, the microorganisms were seeded on nutrient agar and the obtained colonies were used to obtain suspensions in sterile physiological saline corresponding to the 0.5 Mc Farland standard (1-3x108 CFU / mL).

3.5.1. Minimal inhibitory concentration (MIC)

A quantitative method based on performing binary serial microdilutions in liquid medium (nutritive broth) distributed in sterile 96-well plates was used to establish the MIC. A quantity corresponding to a concentration of 5 mg / mL bioactive compound / nanosystem of was added to the first well of each row. Subsequently, with the help of a micropipette, binary dilutions were made, starting from well 1 (concentration 5mg / mL) to well 12 (where the final concentration will be 0.002441406 mg / mL). After microdilution, 15 µL of 0.5 McFarland density microbial suspension was added to each well. The seeded plates were incubated for 24 hours at 37 ° C, and after incubation the MIC value for each compound / nanosystem was established macroscopically, as its last concentration at which no microbial growth was observed, respectively the appearance of environmental turbidity. The MIC was also established by spectrophotometric reading of the absorbance (OD) of the microbial culture developed in the liquid medium at 620 nm (Figure 5)

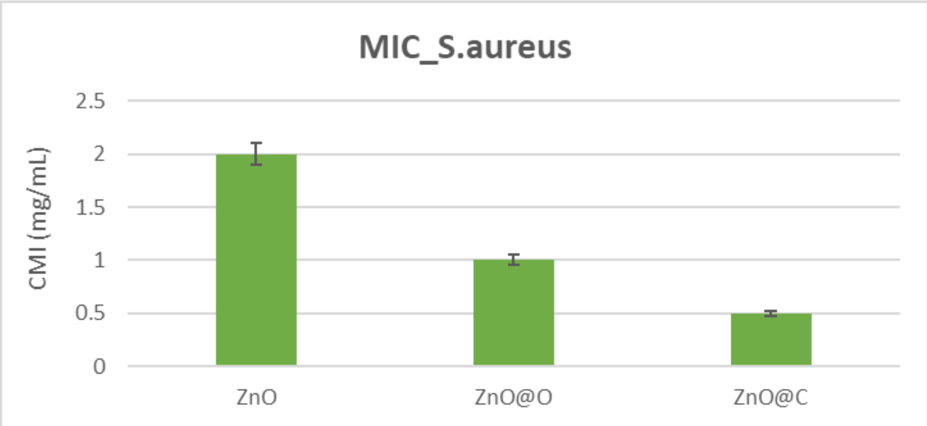


Fig. 5. Minimal inhibitory concentrations obtained for the tested ZnO nanoparticle variants in S.aureus, after 24h of incubation in nutri-tive broth at 37°C.

3.5.2. Biofilm formation

For testing the ability of S.aureus strain to develop monospecific biofilms on the obtained surfaces, the materials were cut to the size of 0.6 cm (discs) and sterilized by expo-sure to UV radiation for 20 minutes on each side. Each piece of sterile material was individually deposited in a well of a 24 sterile wells plate. Over the deposited materials, 1 mL of liquid medium (simple broth) and then 20 μL of 0.5 McFarland density microbial sus-pension were added to the wells. The plates were incubated at 37° C for 24 hours. After incubation, the materials were washed with sterile saline and placed in a sterile tube in one mL of saline. The tube was vortexed vigorously for 30 seconds to detach the cells from the biofilm. The obtained cell suspension was diluted and various dilutions were seeded on plates with solidified culture medium in order to obtain and quantify the number of colony forming units (CFU / mL) (Figure 6).

The antimicrobial results demonstrated that the growth and development of S.aureus is inhibited both in planktonic cultures, by the ZnO nanoparticles, when they are utilized as a suspension, but also in biofilms (when these nanoparticles are embedded into coat-ings). MIC values range 0,5 to 2 mg/mL, the lowest MIC values being obtained for the ZnO@C nanoparticles (figure 5).

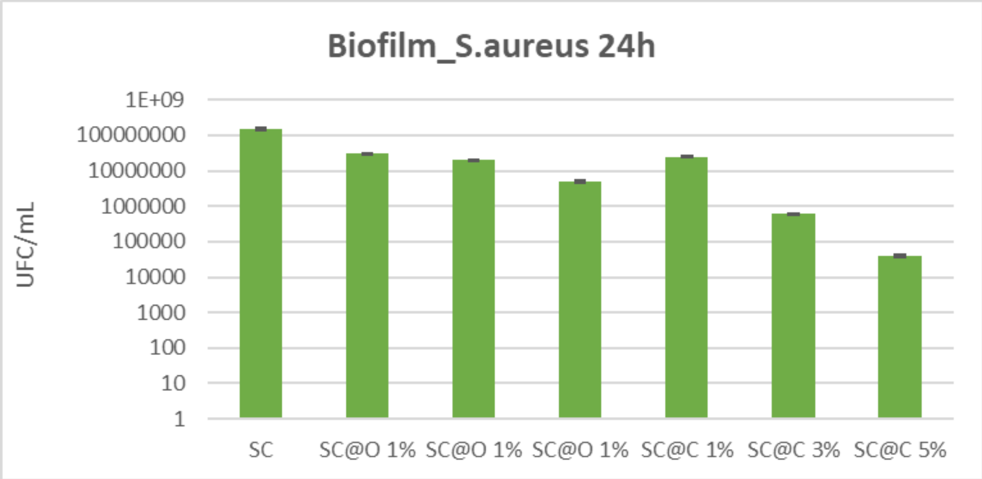


Fig. 6. Graphic representation of the log10 CFU/mL values expressing biofilm cells viability for the S.aureus strain, after 24h of contact with the developed coatings (SC), containing the two types of ZnO nanoparticles in various concentrations (1%, 3% and 5%)

Biofilm formation was also differently altered depending on the ZnO nanoparticle type and their concentration in the developed coatings. Antibiofilm results were consistent with the MIC data, since the greatest biofilm inhibition was obtained for the coatings containing ZnO@C type of nanoparticles (SC@C). A ZnO nanoparticle dose dependent biofilm inhibition was observed for both types of coatings (SC@O and SC@C), even though the SC@C coatings proved a greater S.aureus biofilm inhibition. The SC@C 5% coating material showed the greatest biofilm inhibition, since here we have obtained a 3 fold CFU/mL reduction in the viability of biofilm embedded cells after 24h of incubation (figure 6).

The evaluation of the obtained results shows a good antimicrobial activity of dressings coated with Zinc oxide and essential oils. An increase in functionalized ZnO concentration leads to an increase of the antimicrobial activity.4. Conclusions

Conclusions

The purpose of this study was to synthesize a wound dressing with superior healing properties. Sodium alginate, hyaluronic acid and silk fibroin was used which were coated with nanostructured zinc oxide particles functionalized with pure oregano and clove essential oils.

The morphological analysis shows a porous, scaffold type structure with a very uniform distribution of zinc oxide. The antimicrobial activity was investigated. The tests that were performed show an increase of antimicrobial activity with an increase in functionalized ZnO concentration. The most efficient sample was sodium alginate/hyaluronic acid/ silk fibroin matrix functionalized with 5% ZnO with clove oil.

The obtained samples can be successfully used in the treatment of wounds ensuring a high antimicrobial protection. Due to the materials properties that were used, wound dressing can contribute to a faster healing with no risk of infections in this time.

Acknowledgment

Otilia Ruxandra VASILE and Bogdan Stefan VASILE thanks the Academy of Romanian Scientists for Postdoc Grant and All authors are grateful to Romanian Government through the National Programme “Installations and Strategic Objectives of National Interest”.

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