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Novel hydrogels based on collagen and ZnO nanoparticles with antibacterial activity for improved wound dressings

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Abstract

Hydrogel based wound dressings help to prevent development of infection by absorbing a large volume of exudate from the wound area. Zinc ion based nanoparticles are routinely reported to hold an enhanced potential to help wound healing. In this work, we have developed and characterized hydrogels with in-situ incorporated ZnO nanoparticles (NPs) with antimicrobial properties. Samples were characterized via infrared spectrometry (FTIR), swelling capacity analysis and scanning electron microscopy (SEM). ZnO incorporation lead to an increase in the swallowing rates for tested ZnO concentrations. SEM analysis revealed the presence of agglomerations of ZnO particles of micrometric dimensions (size 1,300-1,315μm) embedded in the polymeric material.The assesment of antimicrobial activity revealed some promising results in case of the liophylised hydrogels incubated with S. aureus The effect of ZnO NPs on biofilm formation was clearly evident in case of S. aureus. The number of viable cells decreased proportionally with the ZnO concentration. The preliminary results highlighted here pave the way for further in vivo experiments in order to confirm the beneficial effects harboured by the novel ZnO hydrogels on host wound healing.

Keywords: Collagen, Zinc oxide nanoparticles, biofilm, wound infection, Antimicrobial activity

1. Introduction

Microorganisms such as *Staphylococcus (S.) aureus*, *Escherichia (E.) coli* and *Candida (C.) albicans* often contaminate, colonize, and infect all types of wounds (BOWLER& al. [1]). Wound infections and possibly high-level colonization represent major barriers to the process of healing (ZHAO & al. [2]). Furthermore, the clustering of microorganisms into biofilms enable the bacteria to gain resistance to the effects of antimicrobial drugs as well as to host defense mechanisms in the wound environment (LAZAR & al. [3]). The presence and negative impact of biofilms in chronic wounds has been increasingly recognized and analyzed over the last years. (PERCIVAL & al. [4]).

Current wound dressings have some major deficiencies including low absorption of wound fluids, low flexibility, a tendency to adhere onto the wound surface, poor mechanical strength and lack of a suitable and moist environment for wound healing. In addition, the majority of current wound dressing do not harbour antimicrobial activity. In this context, hydrogel-based wound dressings would provide a cooling sensation, a moisture environment as well as a barrier to microorganism colonization. Nanoparticles are routinely combined with hydrogels to form hybrid biomaterial systems with antimicrobial activity (WAHID & al. [5]).

Out of the currently available nanoparticles, zinc oxide nanoparticles (ZnO-NPs) have emerged as a powerful antimicrobial tool (EMAMI-KARVANI & al. [6]). A major contributor to antibacterial action is the oxidative stress induced by the ZnO. Importantly, ZnO-NPs were shown to be non-toxic and biocompatible to human cells (PADMAVATHY & al. [7]).

The current study is focused on *in-situ ZnO nanoparticles preparation on collagen matrix as an antibacterial hydrogel for wound dressing* and influence of preparation conditions on material properties, mainly nanoparticles size, shape, homogeneity of their distribution and antibacterial activity.

2. Materials and methods

Preparation of hydrogels based on collagen and ZnO nanoparticles

The present study aims to obtain hydrogels based on collagen and ZnO nanoparticles with antibacterial activity for rapid wound healing, using a *in-situ* synthesis, starting from biocompatible precursors, with low toxicity level. Zinc chloride $ZnCl_2$ 99,999% and ammonia 25% were purchased from Sigma-Aldrich. Bovine collagen type I (0.7%) was produced by SC SANIMED SRL and used as received. All of the other reagents and solvents were of analytical reagent grade and used without further purification. Briefly, the steps for collagen sponges production include collagen extraction from animal tissues by solubilization with non-specific enzymes (5.2% w/w pepsin (250 units/mg) in HCl solution, at a pH value of 1.8), followed by collagen purification using diafiltration (on 10 kDa MWCO membranes) against purified water.

The collagen matrices were weighed and subsequently subjected to cross-linking with 1% glutaraldehyde aqueous solution. Depending on the mass of the collagen matrices, the amount of glutaraldehyde required for cross-linking varied, but in all cases represented 0.5% of the dry mass. The cross-linking process involved placing the collagen matrices in the refrigerator for 24h. After cross-linking, the collagen matrices were subjected to different treatments in order to obtain composite hydrogels with varying ZnO content (2%, 3%, 5%). $ZnCl_2$ was used as a zinc precursor and it was solubilized in 100 ml of distilled water and added in different amounts depending on the dry mass content of each collagen matrix, in order to obtain concentrations of 2%, 3%, 5% ZnO in the final materials. *In-situ* precipitation of ZnO was done by adding a 25% ammoniacal solution, dropwise, into the Zn^{2+} precursor solutions in which the collagen matrices were completely immersed. The obtained matrices were kept at 4° C for 24 hours to complete the reactions and to obtain a better distribution of the nanoparticles throughout the collagen mass. The matrices were subsequently subjected to the lyophilization process (freezing at 55° C for 12 h, vacuum at 0.001 mbar for 12 h and heating under vacuum for 24 hours to 35° C).

Materials characterization

The FTIR spectra were acquired with a spectrophotometer Nicolet iS50R equipped with 3 beam splitters in 12500-50 cm^{-1} range. The FTIR spectra were recorded between 4000- 400 cm^{-1} co-adding 64 scans

at a spectral resolution of $4cm^{-1}$. Morphological aspects of the biocomposite materials were studied via Scanning Electron Microscopy (SEM), with a microscope Quanta Inspect F50 coupled with an energy dispersive spectrometer (EDAX).
Swelling test was performed by immersing samples with $1cm^2$ surface in SBF (simulated body fluid, pH= 7,25) at room temperature to a constant weight. The swollen samples were weighted after wiping out excess SBF from the surface after different time intervals. The swelling properties were determined through the following equation (1):

$$Swelling = (Ws – Wd)/Wd \quad (1)$$

where Ws and Wd are the weights of the hydrogel at swelling state and dry state, respectively.

Antimicrobial activity assay
Qualitative screening for the antimicrobial activity of the obtained hydrogels (disks made of liophilised collagen hydrogels, of 5 mm diameter) was performed using an adapted diffusimetric method. The qualitative screening was tested against standardized microbial strains: *S. aureus* ATCC 6538, *E. coli* ATCC 8739, C. albicans ATCC 10231. The microbial inoculum was made from 18h cultures and and adjusted to a density of 1.5×10^8 CFU/mL, using the 0.5 McFarland standard. The samples for testing were sterilized by UV exposure (30 mins on each side) and placed in contact with the inoculated Petri dishes containing solid media (Müller–Hinton agar for bacteria and Sabouraud agar for yeast). Plates were incubated at 37ºC for 24h to allow microbial development. The level of antimicrobial activity was measured as the diameter of the growth inhibition zones developed around the collagen disks.
To test the effect of the composite hydrogels on biofilm production, the materials (disks of 5 mm diameter) were sterilized by UV exposure for 30 minutes on each side. The fragments of sterile materials were individually placed in 24-well sterile plates. Subsequently, 1 mL of liquid medium (nutrient broth for bacteria and liquid YPG for C.albicans) and 10 μL of 0.5 McFarland microbial suspension were added to the wells. The 24-well plates were incubated at 37°C for 24 hours. After incubation, the materials were washed with phosphate buffer (PBS) and changed in sterile 24 well plates in fresh culture media, which were incubated for 24h to allow biofilm development. Further, samples were washed with PBS and placed in a tube containing 1ml sterile PBS. The tube was vigorously vortexed for 30 seconds and sonicated for 10 seconds, to separate the cells from the biofilms developed on the surface of tested material. The obtained cell suspension was diluted in PBS and various dilutions were seeded on solid culture media plates to obtain and quantify the number of colony forming units/ml (CFUs/ml).

3. Results and Discussions

Hydrogels are an appealing solution to use as wound dressings and fillers. Due to their high water content, gels supply the wound area with a moist, heavily hydrated environment, hence facilitating cellular immunological activity crucial to the wound healing process (VEIGA & al. [8]). Nevertheless, a hydrated environment promotes microbial infection, therefore gels able to convey antimicrobial action in addition to serving their primary functional role (wound healing) are desirable.

Within this study, we prepared *in-situ collagen matrices with ZnO nanoparticles as a novel antibacterial hydrogel for wound dressing*. The obtained hydrogels were analyzed from the point of view of composition, phase interactions and morphology by using FTIR, SEM and antimicrobial activity assays. Figure 1 A depicts the FT-IR spectra for derssings of plain collagen and collagen functionalized with different ZnO concentrations. The absorption bands at 1454, 1404, 1240 and 1205 cm^{-1} of the collagen spectrum are attributed to the stretching vibrations of the CH_2, CH_3, C-N and N-H bonds, respectively. The absorption band at 3291 cm^{-1} corresponds to the collagen specific O-H bond, and it can be observed that, as the ZnO content increases, the absorption band flattens. This can be attributed to a lower number of O-H bonds, due to the collagen's surface modification trying to incorporate ZnO particles. Another visible change in the collagen FT-IR spectrum and therefore in its structure after the addition of 5% ZnO is represented by the small shift of the N-H bond from the 1536 cm^{-1} wavelength to 1529 cm^{-1}, which is another proof of the inorganic-organic interactions from the obtained composites.

In figure 1 B, an increase in swallowing rates can be observed for all samples. The increased swallowing rates are proportional with the increase of time in which the samples remained immersed in SBF (simulated body fluid). The maximum swelling degree was observed 24 hours after immersion, and by comparing the four samples we can say that the greatest swelling capacity was harbored by the collagen dressing with 3% ZnO. The purpose of this assay was to demonstrate the water (i.e. purulent secretion or cloudy fluid that exits the wound) absorption capacity and the ability to maintain a wet environment necessary for healing.

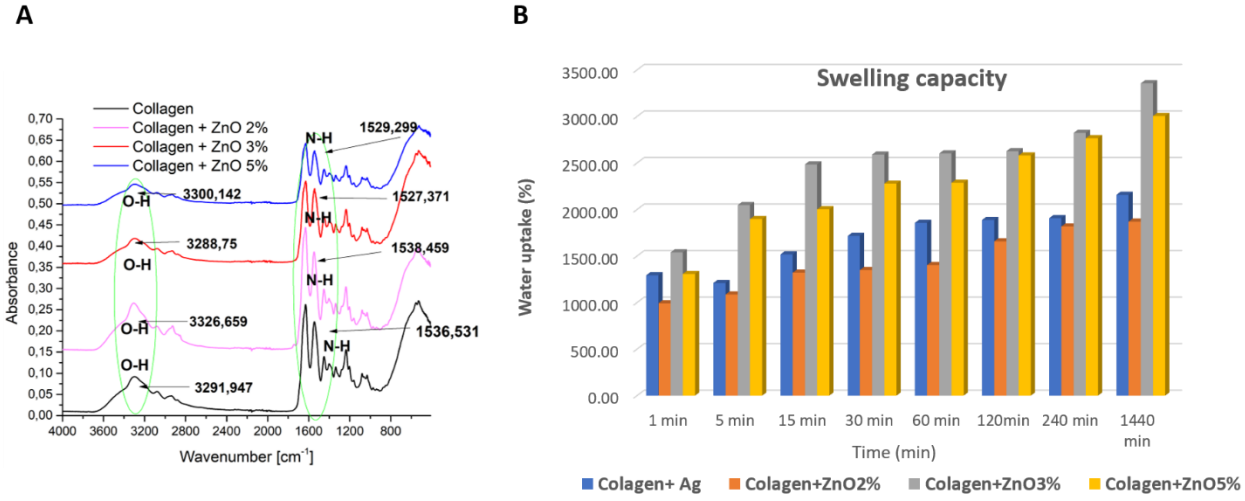

Figure 1. Hydrogels characterization. A. FT-IR spectra for: collagen, collagen-ZnO2%, collagen-ZnO3%, collagen-ZnO5%. **B**. Assessment of swelling capacity

Scanning electron microscopy (SEM) images taken at various magnifications on the collagen sample + 2% ZnO give us information regarding sample morphology (Figure 2 A-C). Figure 2 C (SEM image aquired at magnification 3000x) we can observe an amorphous compound with particle agglomerations and the porous appearance of samples due to the lyophilization process. From a morphological point of view, the micrographs recorded for the collagen sample + 3% ZnO (Figure 2 D-

F) at a magnification of 8000x show the presence of agglomerations of ZnO particles of micrometric dimensions (size 1,300-1,315μm) embedded in the polymeric material.

In the case of micrographs performed on the collagen sample + 5% ZnO, we can see details of the particle size and their arrangement in the collagen matrix (Figure 2 G-I). The size of the recorded agglomerations of particles are between 1,120-1,340 μm. Also, we can observe the collagen as threads with a size of 200-300 nm.

The qualitative assesment of antimicrobial activity revealed some promising results in case of the liophylised hydrogels incubated with *S. aureus* (Figure 3 A, B). All ZnO-functionalized collagen samples lead to growth inhibition of *S. aureus* but the most siginificant effect was harbored by the 2% ZnO-functionalized collagen which led to the highest diameter of bacterial growth inhibtion. Conversely, *E. coli* was not affected by any of the hydrogel samples regardless of the ZnO concentration (data not shown). In line with this, it was reported that excess metal or metal ions can be toxic for bacterial cells. In fact, certain bacteria including *Streptococcus pneumoniae, Haemophilus influenzae, Escherichia coli*, and *B. subtilis* have developed mechanisms to regulate the influx and efflux processes to maintain the steady intracellular concentration of metal ions, including the Zn^{2+} ions (JONES & al.[9]). In case of *C. albicans*, the collagen with 2% Zn O determined a high level of growth inhibition. Interstingly, this effect was not observed in case of higher ZnO concentrations.

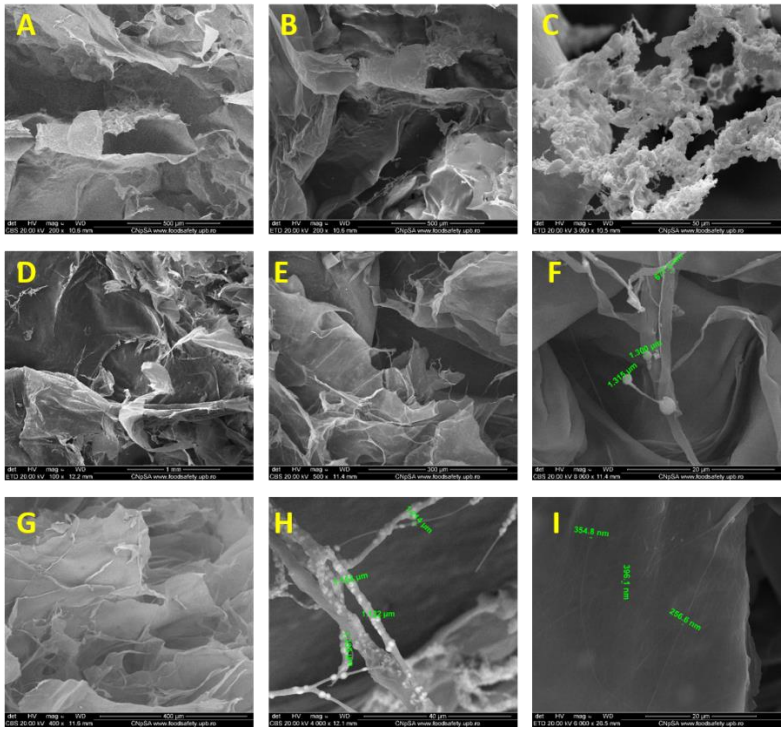

Figure 2. Morphology analysis. Scanning electron microscopy images recorded for collagen + ZnO 2% at different magnifications; 200x (A), 2000x (B), 3000x (C), collagen + ZnO 2% at different magnifications; 100x (D), 500x (E), 8000x (F), collagen + ZnO 5% at different magnifications; 400x (G), 4000x (H), 5000x (I).

The effect of ZnO NPs on biofilm formation was clearly evident in case of *S. aureus*. The number of viable cells decreased proportionally with the ZnO concentration (Figure 3 C). A similar effect was observed in case of *E. coli* (Figure 3D. For *C. albicans* the cellular viability of the biofilm embedded cells was higher in case of the 2% ZnO concentration but similar for the higher ZnO concentrations (Figure 3 E).

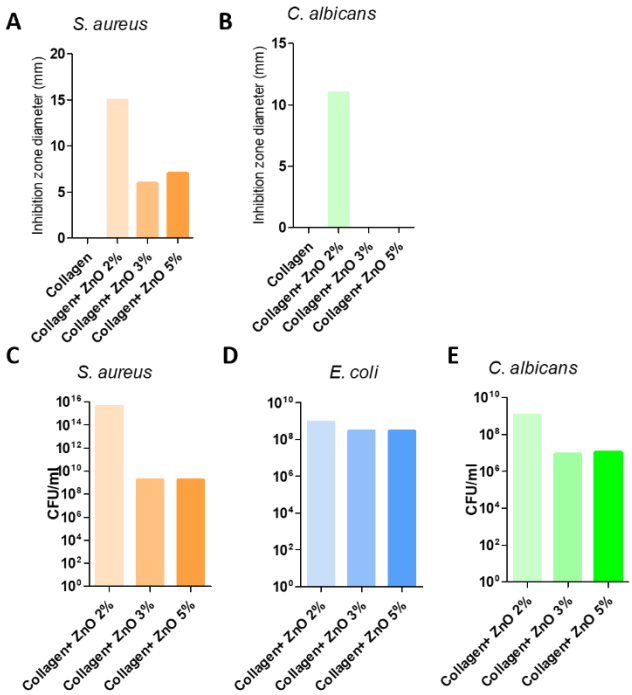

Figure 3. Antimicrobial activity. Qualitative (A, B) and quantitative (C-E) assessment of the antimicrobial activity of ZnO hydrogels

The antimicrobial activity results demonstrate that the obtained collagen dressings could show different biological effects, being more efficient against gram positive bacteria and sometimes yeasts, but less efficient against Gram negative bacteria species. These differences might occur due to the different cell wall structure between Gram positive and Gram negative bacteria. The antimicrobial activity is most likely the result of ROS generation by the released zinc ions from the composite hydrogels. The generated ROS can promote microbial cell wall damage and subsequent death of the microorganism (KUMAR&al [10]). Our results are in accordance with those obtained in other studies. For instance, a recent study by Mohandas et al revealed that ZnO incorporation into alginate hydrogels had an effect on *S. aureus* and *E. coli* but the Zn concentrations used for the antimicrobial activity asay was higher and potentially citotoxic (MOHANDAS &al. [11]). Furthermore, incorporation of ZnO into various matrices such as carboxy methyl cellulose or heparinized polyvynyl alchohol/chitosan lead to the development of antimicrobial activity, a procress would prevent host infection and delayed wound healing (YODALLAHI et al.[12], KHORASANI et al. [13]). However, more investigation is required since high levels of metal ions can be detrimental to host tissues.

4. Conclusions

Wound infections are intrincate by pathogens, and is crucial to note the constantly increasing rates of multiantibiotic resistance among these microorgnasims. For these reasons, wound site infections are a problem for patients and healthcare services. Antimicrobial therapies able to controls colonization and proliferation of microbial pathogens is of paramount importance for skin wound care. Within this line of thought, nanomedicine is a developing field expanding rapidly because of the development of new nanomaterials into a wide range of products and technologies. Zinc oxide nanoparticles harbour low cytotoxicity and ample antibacterial activities, which makes them suitable for the development of alternative products against, for example, multidrug resistant microorganisms. The current study present the development of novel hydrogels containing ZnO in various concentrations followed by their physio-chemical and microbiological characterization. The preliminary results highlighted here pave the way for further *in vitro* and *in vivo* experiments.

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