MANUFACTURING AND CHARACTERIZATION OF GELLAN GUM – ZINC OXIDE COMPOSITES AS POTENTIAL BIOMATERIALS FOR WOUND TREATMENT

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Abstract

This study aimed to produce gellan gum-based hydrogels with the addition of zinc oxide as a potential dressing material. Hydrogels with ZnO concentrations of 0.01%, 0.02% and 0.04% were prepared, micrometric and nanometric ZnO particles were used, and a $CaCl_2$ crosslinker was added to one part of the samples. All samples (14 types) produced by the freeze drying method were characterized with high swelling properties (>2000%), what is important to ensure the absorption of exudates from wounds. Samples with ZnO particles cross-linked with $CaCl_2$ lost less mass after incubation in aqueous media and were characterized by better dimensional stability than those without crosslinking. The pH of the extracts of the samples containing ZnO particles was more neutral (pH 7.0-7.6) than that of the control gellan gum samples (pH of 5.5-6.1). The zinc release from cross-linked samples was twice as high for those containing nanometric particles than for micrometric particles (1.94 ± 0.04 mg/l and 0.93 ± 0.02, respectively). Relatively large amounts of released zinc species in the case of samples containing ZnO nanoparticles are promising in the context of the antibacterial properties and treatment of infected wounds. A lower amount of zinc released from samples with ZnO microparticles could be sufficient to prevent the development of the infection. Furthermore, both materials show satisfactory cytocompatibility with L929 fibroblasts, as shown by Alamar blue and live/dead viability tests, making them prospective candidates for wound healing.

Keywords: *gellan gum, zinc oxide nanoparticles, zinc oxide microparticles, wound healing, L929 fibroblasts*

Introduction

Hydrogels are materials widely used as wound dressings. Due to their high water content, they provide a moist environment that promotes wound healing and also allows gas exchange. Hydrogels adhere very well to the wound, thus preventing contamination of damaged tissue, and at the same time, they do not stick to it, so changing the hydrogel dressing is painless [1]. In addition, especially when hydrogels are processed by freeze-drying to form porous sponges, they can absorb exudate from the wound and cleanse it of necrotic tissues [2]. Hydrogels are usually formed by synthetic polymers, e.g., poly(vinyl alcohol) (PVA), poly(vinyl pyrrolidone) (PVP) and poly(ethylene glycol) (PEG) or by natural polysaccharides such as starch, cellulose, chitosan, agar, alginates or gellan gum [3].

Gellan gum is a biodegradable and nontoxic polysaccharide, obtained in the fermentation process by *Sphingomonas elodea* bacteria [4]. It is used in the food industry because of its gelling properties and as a stabiliser of suspensions; it is also applied in the medical industry, for example, in tissue engineering as a matrix of scaffolds or as a drug carrier [5,6]. Gellan gum hydrogels are most often cross-linked using calcium ions [7]. Gellan gum-based materials supplemented with lipid nanoparticles loaded with antibacterial peptide, nisin, have already been developed as potential dressing materials for infected wounds [8].

Zinc oxide is known for its antibacterial properties that may have a beneficial effect on wound healing [9]. The antibacterial effect of ZnO is related to the production of reactive oxygen species (ROS) that destroy the bacterial cell wall. It can also release zinc ions, Zn^{2+}, which are absorbed by the bacterial cell, damaging DNA, and reacting with intracellular proteins [10]. ZnO is also a potential cross-linker, improving the mechanical properties of polysaccharide hydrogels, as well as increasing resistance to degradation [11].

The purpose of this study was to develop dressing materials based on gellan gum supplemented with zinc oxide particles. To this end, several sample types were manufactured that differed in ZnO particle size (nanometric or micrometric) and particle concentration. Then, physico-chemical properties of the samples including dimensional stability, swelling properties, and zinc release were assessed. The study also aimed at checking the cross-linking properties of ZnO powders depending on the concentration and particle size and its effect on the properties of the resulting materials. In addition, cytotoxicity tests with the use of L929 fibroblasts of all studied materials were performed to find out which has the most promising properties for application as wound dressings.

Materials and Methods

Preparation of gellan gum samples with zinc oxide particles

Gellan gum (GG, low-acyl, Sigma Aldrich, USA), nanometric ZnO (<100 nm particle size, Sigma-Aldrich, USA) and micrometric ZnO (<50 µm particle size, ZM Silesia SA, Oława, Poland) were used in this study.

The GG control sample was prepared by dissolving 200 mg of GG in 10 ml of UHQ ultrapure water for 30 min in a 90°C water bath. The suspension was vortexed for 10 s and poured into a glass Petri dish (diameter 9 cm). Spacers placed in a Petri dish made it possible to obtain a uniform hydrogel height of 4 mm.

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The cross-linked GG control sample (GG+$CaCl_2$) was prepared by dissolving 222 mg of GG in 10 ml of ultrapure UHQ water for 30 min in a 90°C water bath. The temperature was then reduced to 70°C and the crosslinking solution (1% $CaCl_2$ solution) was heated in a water bath: 493 mg $CaCl_2 \cdot 6H_2O$ in 24.76 ml UHQ water. Then 9 ml of the GG suspension and 1 ml of the crosslinking solution were taken, vortexed for 10 s and poured into a Petri dish (diameter 9 cm) with spacers. The final concentration of $CaCl_2$ in the samples was 0.1%.

The GG samples with the addition of ZnO at concentrations of 0.01 wt%, 0.02 wt%, and 0.04 wt% were also prepared by weighing the appropriate amount of ZnO (1.1 mg, 2.2 mg, 4.4 mg, respectively), which was suspended in 11 ml of UHQ water using ultrasound for 30 min. 220 mg of GG were weighed and mixed with ZnO suspension, then heated for 30 min in a water bath at 90°C. 10 ml of suspension was taken, vortexed for 10 s, and poured into a Petri dish (diameter 9 cm) with spacers. Two ZnO powder grades were used: nanometric (n) and micrometric (m), resulting in six sample types: GG+ZnO(n)0.01%, GG+ZnO(n)0.02%, GG+ZnO(n)0.04%, GG+ZnO(m)0.01%, GG+ZnO(m)0.02%+$CaCl_2$, GG+ZnO(m)0.04%.

The GG+$CaCl_2$ samples with the addition of ZnO at concentrations of 0.01 wt%, 0.02 wt%, and 0.04 wt% were also prepared by weighing the appropriate amount of ZnO (1.1 mg, 2.2 mg, 4.4 mg, respectively), which was suspended in 11 ml of UHQ water using ultrasound for 30 min. 220 mg of GG were weighed and mixed with ZnO suspension, then heated for 30 min in a water bath at 90°C. Then, 9 ml of the suspension and 1 ml of 1% $CaCl_2$ were taken, vortexed for 10 s, and poured into a Petri dish (diameter of 9 cm) with spacers. Two ZnO powder grades were used: nanometric (n) and micrometric (m), resulting in six sample types: GG+ZnO(n)0.01%+$CaCl_2$, GG+ZnO(n)0.02%+$CaCl_2$, GG+ZnO(n)0.04%+$CaCl_2$, GG+ZnO(m)0.01%+$CaCl_2$, GG+ZnO(m)0.02%+$CaCl_2$, GG+ZnO(m)0.04%+$CaCl_2$.

The hydrogels were cooled at 4°C for 30 min, and then circular samples with a diameter of 12 mm were cut out with a punch device. The hydrogel samples were placed in 24-well plate dishes, frozen at -80°C for 24 h followed by 48 h freeze-drying (Alpha 1-2, Martin Christ, Osterode am Harz, Germany).

Microscopic observations

The freeze dried samples were observed under a microscope (Keyence VHX 7000, Belgium) at a magnification of 20x. Each of the samples was then immersed in 3 ml of phosphate buffered saline (PBS, Sigma-Aldrich, USA) for 1 h, after which microscopic observations were made again to assess microstructural changes after incubation in PBS.

Swelling, remaining mass, pH measurements

The freeze dried samples (n = 3 of each type) were weighed on an analytical balance and then immersed in 2 ml of PBS each. After 24 h, the samples were collected, and carefully wiped with a tissue (Kimwipes, KIMTECH) to remove excess water, but avoiding removing water out of the pores of the sample.

The swelling of the samples was calculated from the formula (1):

$$\%\text{Swelling} = \frac{M_w - M_d}{M_d} \cdot 100\% \qquad (1)$$

where: M_w - the mass of the wet sample, M_d - the mass of the dry sample.

After 48 h of incubation in PBS, the samples were washed extensively with UHQ water (4 times, 5 ml of water, 3 min for each wash) and dried at 37°C for 24 h. The dried samples were weighted. The remaining mass of the samples was calculated using the formula (2):

$$\%\text{Remaining mass} = \frac{M_{deg}}{M_{non\text{-}deg}} \cdot 100\% \qquad (2)$$

where: M_{deg} is the mass of the dried sample after 48 h of incubation, $M_{non\text{-}deg}$ is the mass of the dry sample before incubation.

For pH measurements of the incubation medium, previously weighed samples were placed in separate Falcon tubes, and about 3 ml of UHQ water was poured into each tube so that the ratio of sample weight to water volume was constant in each tube and equal to 0.425%. The pH of the samples was measured after 24 h (Elmetron 410 pH Meter, Poland). The pH meter was thoroughly cleaned between measurements. To test the stability of the samples, they were incubated in 3 ml of UHQ water for 7 days. After 7 days, it was checked whether the hydrogels structure changed or degraded.

Atomic absorption spectrometry

To determine the ZnO release from the hydrogel samples, atomic absorption spectrometry (Perkin-Elmer 3110, USA) was used. Extracts from hydrogel samples after 10 days of incubation in water (the same as those used for the pH measurements) were used for the analysis. The suspensions were centrifuged and 0.5 ml of sample solution of the respective samples was added: GG, GG+$CaCl_2$, GG+ZnO(n)0.04%, GG+ZnO(n)0.04%+$CaCl_2$, GG+ZnO(m)0.04%, GG+ZnO(m)0.04%+$CaCl_2$.

In vitro cytotoxicity

To test potential cytotoxicity, 1% extracts of the samples were prepared. For this purpose, two samples of each type were weighed. Each sample was irradiated under a UV lamp for 10 min on each side. The samples were placed in Falcons tubes and incubated in cell culture medium (the same as for further *in vitro* tests, 2 ml of medium for 20 mg of samples) for 24 h.

L929 fibroblasts (European Collection of Cell Cultures, UK) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin (all chemicals from PAN-Biotech) at 37°C in a 5% CO_2 atmosphere. A suspension of 50,000 cells/ml of medium was prepared. In a 96-well plate, 200 µL of cell suspension was transferred to each well resulting in 10,000 cells per well and allowed to adhere to the bottom of the plate. After 24 h, the medium was aspirated and replaced with extracts of the samples. Cells were cultured in 1% and 0.5% w/v extracts of the samples as well as in control medium without extracts (n = 3).

Cell viability was tested with the AlamarBlue assay [12]. After 24 h of cell culture with extracts, the medium was aspirated from each well and replaced with 150 µl of a 5% AlamarBlue solution in DMEM. The prepared plate was incubated for 3 h and then 100 µl of medium was transferred to a 96-well black plate. Using the FluoroSTAR Omega reader, the fluorescence intensity of the samples was obtained. The percentage of resazurin reduction was calculated using the formula (3):

$$\%\text{Resazurin reduction} = \frac{F_x - F_{0\%}}{F_{100\%} - F_{0\%}} \cdot 100\% \qquad (3)$$

where: F_x - fluorescence of the sample, $F_{0\%}$ - fluorescence of the medium with AlamarBlue reagent without cells, $F_{100\%}$ - fluorescence of the completely reduced reagent.

For live/dead staining, the extracts were replaced with a solution of 100 µl of 0.1% calcein AM (Sigma-Aldrich) and 0.1% propidium iodide (Sigma-Aldrich) solutions in PBS. The plate was incubated for 20 min at 37°C and then a fluorescence microscope (Axiovert 40 CFL, Germany) was used for cell visualization.

Statistics

Statistical analysis was performed by one-way analysis of variance (one-way ANOVA) using OriginLab2023 software. Differences were considered significant when $p < 0.05$. The results are presented as mean ± standard deviation (SD).

Results and Discussion

During hydrogel production, it was observed that with an increase of the ZnO concentration, the samples became stiffer and harder, and the highest stiffness was found for the samples with 0.04% ZnO and $CaCl_2$ cross-linker. The control sample of neat gellan gum was the softest, so great care had to be taken when cutting the GG hydrogels without damaging the samples.

Microscopic observations

The results of microscopic observations of dry samples and those after soaking in PBS for 1 h (wet) are shown in FIG. 1. With the increase in the concentration of ZnO, whiter colouration of the samples was observed. Samples with the addition of $CaCl_2$ cross-linker had larger pores compared to samples without $CaCl_2$. The sample of ZnO(n) 0.04%+$CaCl_2$ had the largest pores. The highest porosity was found for samples containing nanometric ZnO with the addition of a $CaCl_2$ crosslinker. After soaking in PBS, the size of the samples increased (FIG. 1).

Swelling, remaining mass, pH measurements

The swelling of the samples after 24 h of incubation in PBS was very high for all samples always exceeding 2000% (TABLE 1, column 2).

The results of the samples' mass measurements after 48 h incubation in PBS (TABLE 1, column 3) show that the addition of ZnO to the non-cross-linked samples caused a higher mass reduction than in the control GG sample. The lowest remaining mass equal to 75% was measured for GG+ZnO(n)0.04% sample. Samples cross-linked with $CaCl_2$ showed a much higher remaining mass, except GG+ZnO(m)0.01%+$CaCl_2$ for which the remaining mass was 87%.

The pH measurements (TABLE 1, column 4) show that each sample containing ZnO caused an increase in pH as compared to the GG and GG+$CaCl_2$ control samples which presumably is due to the release of zinc species from the hydrogels into the solution. The samples with the addition of ZnO showed a pH of 7.0-7.6, while the samples of GG and GG+$CaCl_2$ had a pH of 5.5 and 6.1, respectively.

The last column of TABLE 1 shows the dimensional stability of the hydrogels. The "yes" mark means that the sample did not degrade within 7 days of incubation in UHQ water, while the mark "no" means that the sample degraded under these conditions.

The results show that all samples crosslinked with $CaCl_2$, except GG+ZnO(m)0.01%+$CaCl_2$, were dimensionally stable up to 7 days immersion in UHQ water. The latter sample was also characterized by a higher loss of mass of 13% and a very high swelling that reached 3700 ± 300%. On the other hand, the only one sample without $CaCl_2$, i.e. GG+ZnO(m)0.04%, was dimensionally stable after 7 days of incubation. It had a relatively low swelling of 2250 ± 300% and lost only 8% of its mass after incubation. The other samples without $CaCl_2$ crosslinking dissolved within 7 days of incubation.

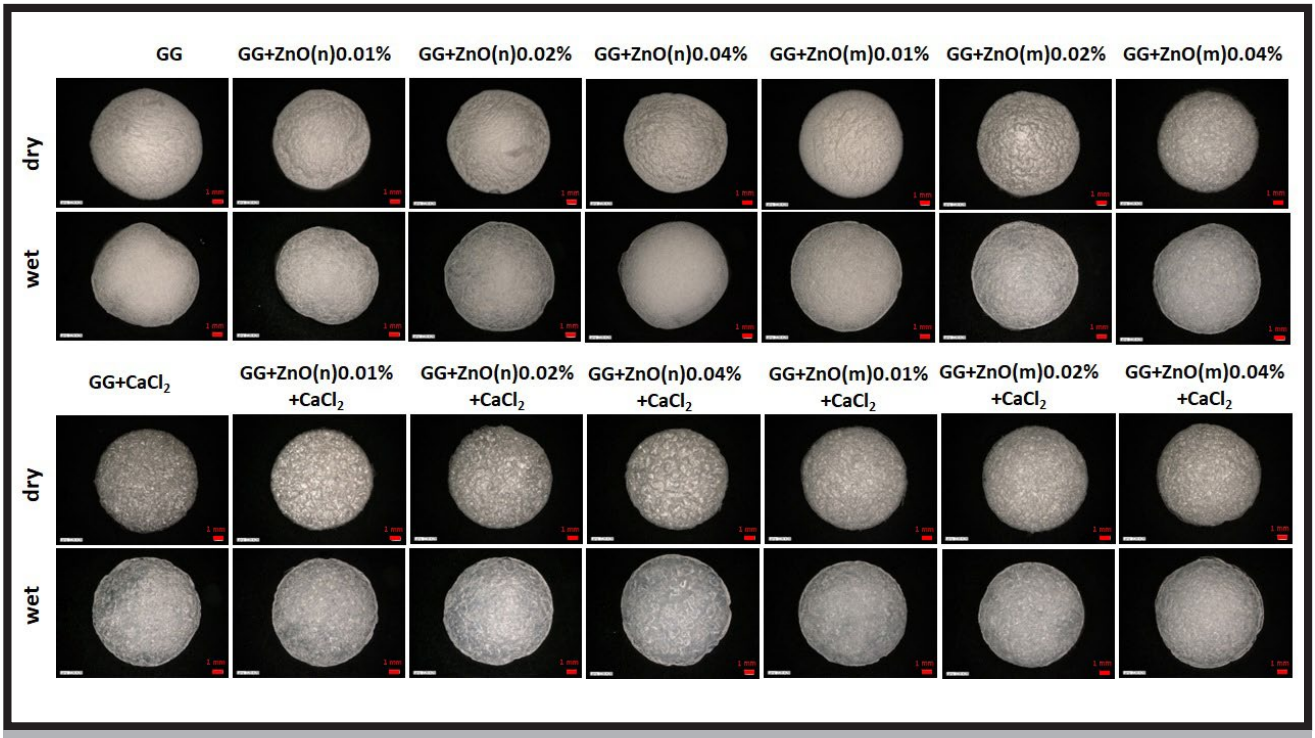

FIG. 1. Pictures of samples after freeze-drying (dry) and after soaking in PBS for 1 h (wet) (Keyence VHX 7000, Belgium, 20x magnification); scale bar = 1 mm.

TABLE 1. Swelling (after 24 h in PBS), remaining mass (after 48 h in PBS); pH value (after 24 h incubation in UHQ water), and dimensional stability of the samples (after 7 days in UHQ water).

Sample	Swelling [%)]	Remaining mass [%]	pH	Dimensional stability
GG	2500 ± 600	97	5.5	no
GG+ZnO(n)0.01%	3200 ± 100	92	7.5	no
GG+ZnO(n)0.02%	3200 ± 100	94	7.5	no
GG+ZnO(n)0.04%	2950 ± 150	75	7.6	no
GG+ZnO(m)0.01%	2100 ± 200	88	7.5	no
GG+ZnO(m)0.02%	2500 ± 700	92	7.6	no
GG+ZnO(m)0.04%	2250 ± 330	92	7.2	yes
GG+$CaCl_2$	3050 ± 400	93	6.1	yes
GG+ZnO(n)0.01%+$CaCl_2$	2340 ± 80	98	7.0	yes
GG+ZnO(n)0.02%+$CaCl_2$	3900 ± 500	104	7.1	yes
GG+ZnO(n)0.04%+$CaCl_2$	3500 ± 400	103	7.3	yes
GG+ZnO(m)0.01%+$CaCl_2$	3700 ± 300	87	7.3	no
GG+ZnO(m)0.02%+$CaCl_2$	2700 ± 300	98	7.2	yes
GG+ZnO(m)0.04%+$CaCl_2$	2900 ± 100	93	7.2	yes

The results obtained show that cross-linked samples with 0.04% ZnO particles (both micrometric and nanometric) and non-cross-linked with 0.04% micrometric ZnO seem to be the most promising for dressings due to their dimensional stability. Hence, they were submitted to further studies to assess zinc release and verify their potential cytotoxicity. For comparison, GG+ZnO(n)0.04%, which was not dimensionally stable and lost the highest amount of mass after incubation, was also tested.

Atomic absorption spectrometry

The results of atomic absorption spectrometry results of zinc release after 10 days of incubation in UHQ water for the samples GG+ZnO(n)0.04%, GG+ZnO(m)0.04%, GG+ZnO(n)0.04%+$CaCl_2$, and GG+ZnO(m)0.04%+$CaCl_2$ are shown in FIG. 2. For the above samples, the corresponding zinc concentrations were equal to 2.51 ± 0.04 mg/l, 0.92 ± 0.03 mg/l, 1.94 ± 0.04 mg/l, and 0.93 ± 0.02 mg/l. There was a significantly higher amount of zinc released in the case of the samples with nanometric ZnO compared to that with micrometric ZnO. It may be related to a higher dissolution of ZnO from nanoparticles compared to microparticles due to their higher relative surface area. Crosslinking of the hydrogel with Ca^{2+} ions decreased the amount of zinc released from the composites but only in the case of nanometric ZnO particles. No such relationship was found for micrometric ZnO particles, for which zinc release was the lowest. For comparison, GG and GG+$CaCl_2$ samples were also tested, but zinc, as expected, was not detected in these samples. Our results show that the amount of zinc released should be sufficient to assure antibacterial properties. According to Jayaseelan et al. [13] minimum inhibitory concentrations (MIC) of ZnO nanoparticles against *A. hydrophila, E. coli, E. faecalis, C. albicans* were 1.2, 1.2, 1.5, and 0.9 µg/ml, respectively. According to Ahmed Kadhum [14] the MIC of ZnO nanopatricles against *P. aeruginosa, K. pneumoniae, E. coli, S. aureus, S. epidermidis* and *S. pneumoniae* was reported to be 1.25 µg/ml.

In vitro cytotoxicity tests

The resazurin reduction test assessing cell viability was carried out for the control sample (L929 cells cultured in the medium, without the addition of extracts) and the hydrogel extracts at a concentration of 0.5% and 1% (FIG. 3). Similar viability was obtained for GG and GG+$CaCl_2$ samples for both 0.5% and 1% extracts. In the case of the 0.5% and 1% extract for GG, the degree of reduction of Alamar Blue was 45.1 ± 2.5%, 42.0 ± 6.5%, respectively. For the GG+$CaCl_2$ 0.5% and 1% extracts, it was 48.8 ± 0.1% and 42.3 ± 1.3%, respectively. The control sample showed viability of 66.4 ± 5.9%.

For all samples containing ZnO particles, cell viability was higher in the presence of 0.5% extracts than in the case of 1% extracts. For a non-cross-linked sample containing ZnO nanoparticles (i.e. GG+ZnO(n)0.04%), for which the amount of zinc released was the highest (FIG. 2), and which was also the least stable during incubation (TABLE 1), the viability of the cells was also the lowest.

Interestingly, a level of cell viability comparable to that of the control sample was observed for the samples GG+ZnO(n)0.04%+$CaCl_2$ and GG+ZnO(m)0.04%+$CaCl_2$, i.e., crosslinked with calcium ions, for the 0.5% extract, which were 72.5 ± 6.9% and 74.5 ± 1.6%, respectively. It shows a great potential of $CaCl_2$ cross-linked samples supplemented with 0.04% ZnO particles for biomedical applications, provided that the concentration of particles and release of zinc are at a defined, not too high level, which is not toxic to cells. For our samples, the highest concentration of zinc released was 2.51 ± 0.04 mg/l for GG+ZnO(n)0.04% sample (FIG. 2). According to the literature, the half-maximum inhibitory concentration (IC50) of ZnO for L929 cells was found to be 50 µg/ml (50 mg/l) [15]. No negative influence of ZnO on HeLa cells was reported for the concentration of ZnO not exceeding 20 mg/l [15].

For 0.5% extracts of GG+ZnO(n)0.04%+$CaCl_2$ and GG+ZnO(m)0.04%+$CaCl_2$ samples, there are no statistically significant differences as compared to control.

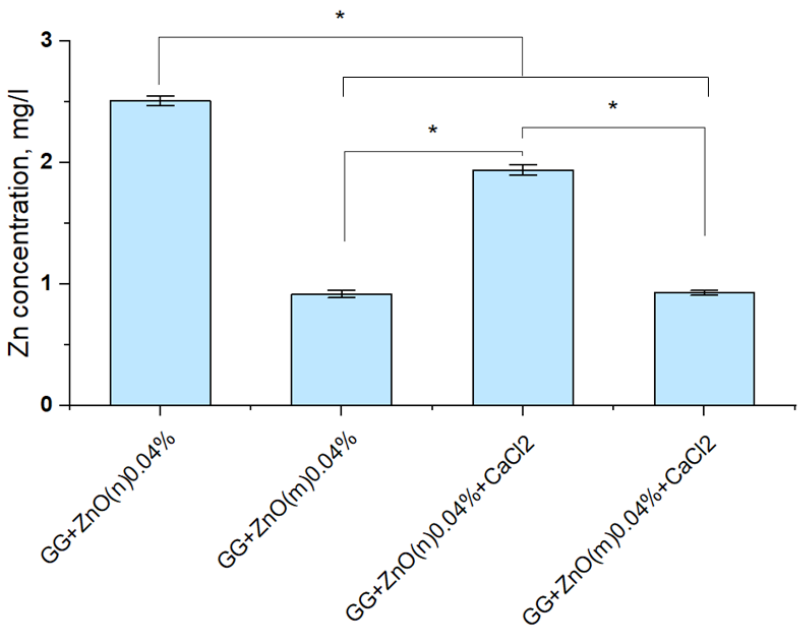

FIG. 2. Zinc concentration in the aqueous medium after immersion of the samples evaluated by atomic absorption spectrometry. Average from n = 3 samples ± SD (standard deviation), *p < 0.05.

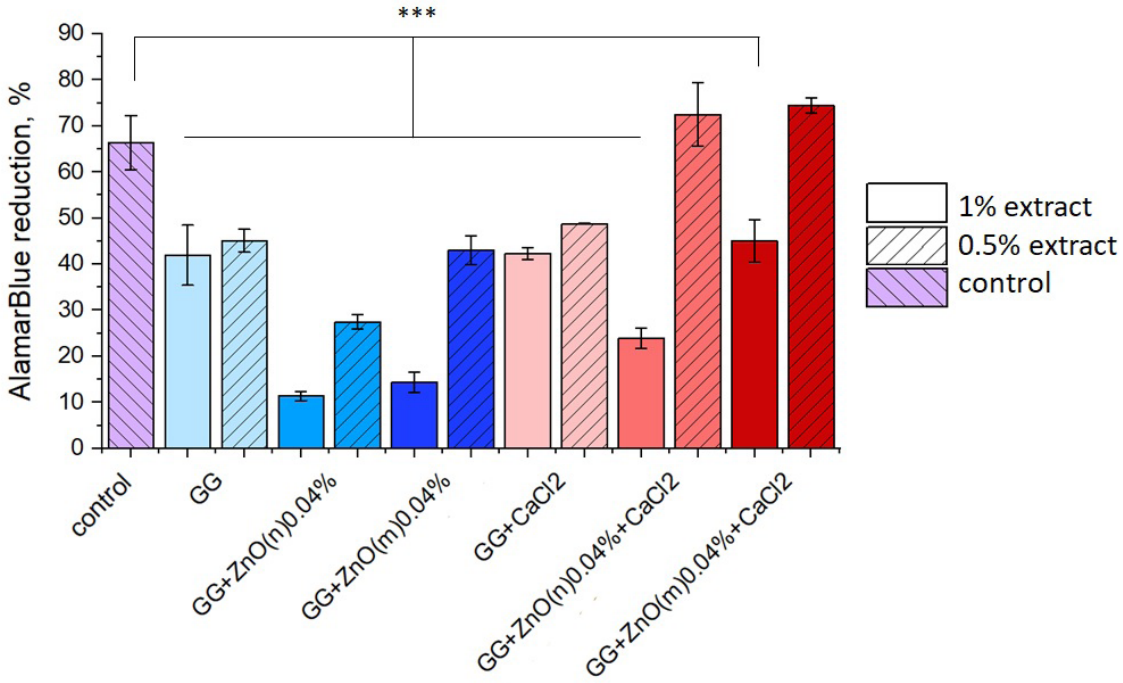

FIG. 3. Viability of L929 cells in contact with 1% and 0.5% extracts from the samples and cells cultured in control conditions (medium without extract addition). Average from n = 3 samples ± SD (standard deviation), ***p < 0.001.

Fluorescence microscopy images of live/dead stained cells cultures in 0.5% and 1% extracts from the samples are shown in FIG. 4.

Live/dead images show the high viability of L929 cells, in particular, for 0.5% hydrogel extracts. The least viable cells are visible in the pictures with 1% hydrogel extracts of GG+ZnO(n)0.04% and GG+ZnO(m)0.04% without the addition of $CaCl_2$ cross-linker. The results suggest that the absence of this cross-linker caused a greater release of ZnO into the extract, which could have a toxic effect on the cells. This hypothesis is confirmed by the results of the resazurin reduction test, where the lowest percentage of cell viability was observed in the case of samples not containing $CaCl_2$. The 1% extracts were more toxic to cells than the 0.5% extracts, showing that it is possible to reduce the potential cytotoxicity of the materials by reducing the amount of ZnO added and using the particles with micrometric size instead of nanometric size.

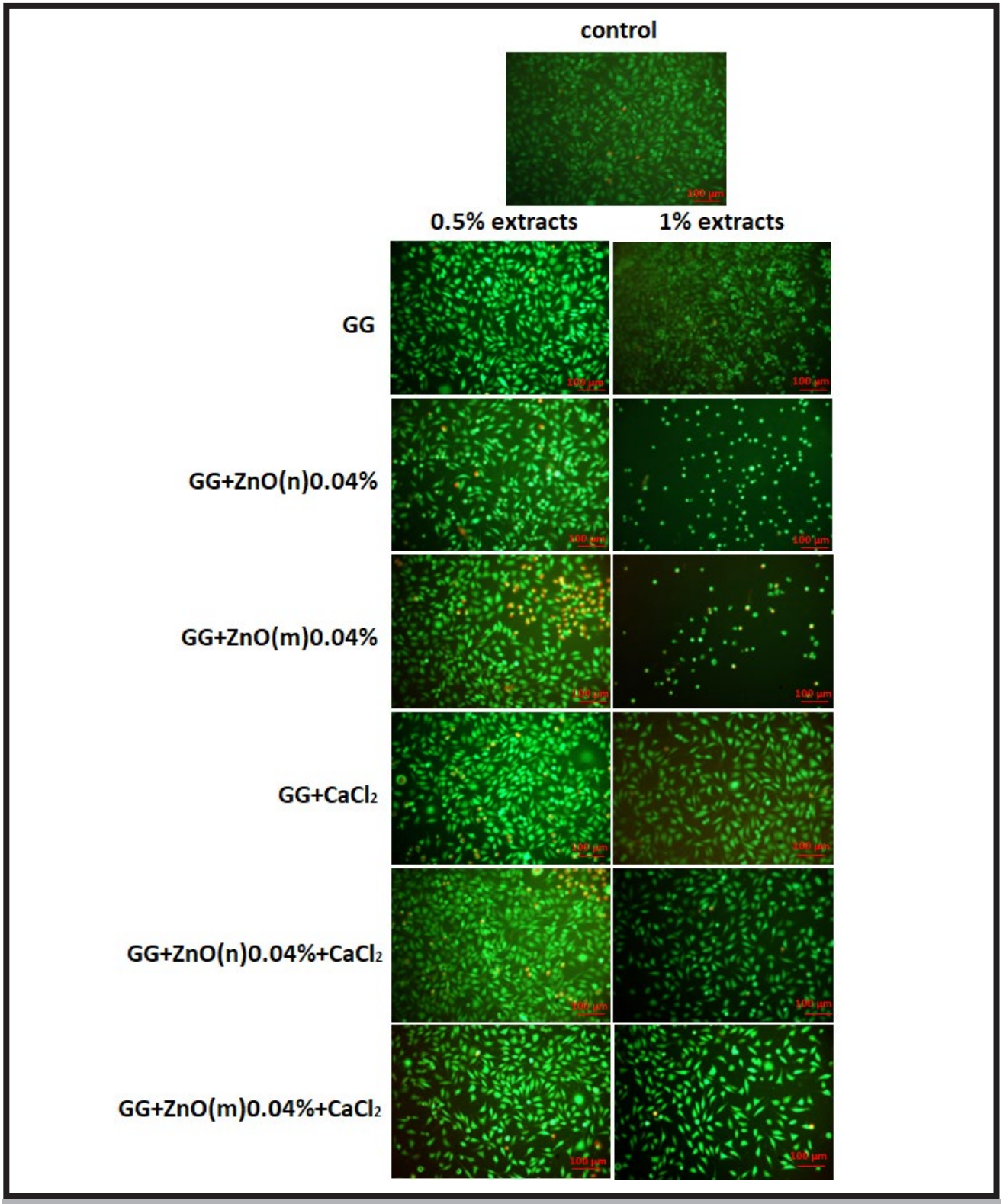

FIG. 4. Live/dead images of L929 cells in contact with 1% and 0.5% extracts from the samples and cells cultured in control conditions (medium without extract addition). Scale bar = 100 µm.

Conclusions

The study aimed at producing composite hydrogels with the addition of zinc oxide in the form of nanometric and micrometric particles as an antibacterial agent and an additional cross-linker that influences several physicochemical properties of the samples (stiffness, porosity, swelling properties, stability, etc.) and biological performance with L929 fibroblasts.

Microscopic examinations showed a higher porosity of the samples containing $CaCl_2$ compared to the samples without this cross-linker. All samples were characterized by high swelling, which is important to ensure absorption of exudates from wounds. Samples with ZnO particles cross-linked with $CaCl_2$ lost less mass after incubation in aqueous media and were characterised by better dimensional stability than those without crosslinking. The pH of the extracts of the samples containing ZnO particles was more neutral than that of the control gellan gum samples.

The release of zinc was higher from the samples containing nanometric particles than in the case of micrometric particles. This may be due to an increase in the solubility of ZnO along with a decrease in particle size, because of the higher relative surface area, which resulted in a greater degree of transfer of zinc from the samples to aqueous medium. Interestingly, it was found that zinc release can also be controlled by cross-linking of the gellan gum matrix, because for cross-linked samples containing ZnO nanometric particles, the release of zinc was reduced.

Relatively large amounts of zinc species in the case of samples containing ZnO nanoparticles (GG+ZnO(n)0.04% +$CaCl_2$) are very promising in the context of antibacterial properties and treatment of infected wounds. The lower amount of zinc released in the case of the samples with ZnO microparticles (GG+ZnO(m)0.04%+$CaCl_2$) might be sufficient to prevent infection development. Furthermore, both materials show satisfactory cytocompatibility with L929 fibroblasts and durability for at least 7 days under aqueous conditions, paving the way for their use as dressings for infected wounds.

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