DEVELOPMENT AND CHARACTERIZATION OF SODIUM ALGINATE-ZnO NANOPARTICLE REINFORCED COMPOSITES FOR BIOMEDICAL APPLICATIONS

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The field of pharmaceutical science is witnessing a surge in interest in developing controlled-release dosage forms to optimize therapeutic outcomes. Biodegradable polymers like chitosan, sodium alginate, and starch offer significant advantages due to their biocompatibility and ability to form diverse drug delivery systems. Alginate, derived from brown seaweed or bacterial sources, is particularly promising for its antimicrobial properties. This study aims to evaluate the characteristics of a porous composite material comprising alginate (Alg) as a matrix and microwave-assisted hydrothermal synthesized zinc oxide nanoparticles (ZnO NPs) as a reinforcing agent. Leveraging the distinctive properties of both alginate and ZnO NPs, this research seeks to develop a material with prospective antimicrobial efficacy and reduced cytotoxicity, potentially applicable in biomedical fields, particularly for advanced wound dressing solutions. The innovation in this study also stems from incorporating salicylic acid (SA) into the proposed Alg-ZnO wound dressings, addressing critical aspects such as infection control, inflammation reduction, and tissue regeneration promotion. The obtained materials were characterized morphologically and spectroscopically, as well as tested to establish cytotoxicity and biocompatibility. Thus, SEM/EDS and FTIR analysis indicated a good structural integrity and high functional performance of the composites. XTT and LDH assays indicated high cellular viability and a minimal cell membrane damage, which recommends them for biomedical applications.

Keywords: ZnO nanoparticles, sodium alginate, salicylic acid, microwave-assisted hydrothermal synthesis, cellular viability

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1. Introduction

The field of pharmaceutical science has seen a burgeoning interest in the development of innovative controlled-release dosage forms. These advanced formulations aim to optimize therapeutic outcomes by reducing dosing frequency and extending the duration of drug efficacy. Achieving these objectives necessitates the selection of appropriate excipients that are both effective and biocompatible. Among the most extensively studied materials for this purpose are biodegradable polymers, including chitosan, sodium alginate, and starch. These polymers offer significant advantages due to their inherent biodegradability, biocompatibility, and ability to form diverse drug delivery systems that can be tailored to meet specific clinical needs [1-2]. Moreover, materials derived from natural resources have become increasingly important in biomedical applications, particularly for evaluating, replacing, treating, or augmenting skin [3], tissues, organs, or functions compromised by diseases or trauma [4]. Incorporating therapeutic agents into polymeric matrices can significantly enhance the protection of bioactive compounds from degradation, precisely control their release profiles, and improve their absorption. Additionally, these strategies can help mitigate the high costs associated with conventional therapeutic approaches.

Alginate has emerged as a promising biopolymer for further research due to its versatile properties and natural origin. Commercially available alginate is derived primarily from the cell walls of brown seaweed (*Phaeophyceae*) or from bacterial sources (*Laminaria hyperborea, Laminaria digitata, Laminaria japonica, Ascophyllum nodosum)*, which, through a series of treatments involving acidification, alkaline extraction, purification processes, and conversion, yields a water-soluble sodium alginate powder [5].

Alginate's utility spans various fields, including medicine, pharmacy, and environmental quality control [6], with demonstrated antimicrobial properties [7]. Moreover, incorporating nanoparticles effective against pathogens, such as zinc oxide nanoparticles (ZnO NPs), into the alginate matrix can enhance these antimicrobial effects. ZnO NPs are recognized by the US Food and Drug Administration as safe and non-toxic to human health [8]. Various synthesis methods for ZnO NPs are documented in the literature. Among them, the microwave-assisted hydrothermal synthesis method recently gained attention for its efficiency in producing nanoparticles with uniform size, shape, and distribution [8]. Microwave irradiation offers a rapid and efficient heating mechanism, significantly expediting the synthesis process compared to traditional methods.

This study aims to evaluate the characteristics of a porous composite material consisting of alginate (Alg) as a matrix and microwave-assisted hydrothermal synthesized ZnO NPs as a reinforcing agent. By leveraging the unique properties of both alginate and ZnO NPs, this research seeks to develop a

biocompatible material with prospective antimicrobial efficacy and potential applications in biomedical fields, particularly for advanced wound dressing solutions. Moreover, salicylic acid (SA) was added to some Alg-ZnO formulations. Based on the literature review, incorporating SA into the proposed wound dressings might assess key aspects such as infection control, inflammation reduction, and promotion of tissue regeneration [9-10]. These properties, combined with the ability to enhance the efficacy of other therapeutic agents, make salicylic acid a valuable addition to the proposed wound care formulations.

2. Materials and Methods

2.1 Materials

Sodium alginate (alginic acid sodium salt from brown algae), zinc chloride ($ZnCl_2$, p > 98%), calcium chloride ($CaCl_2$, anhydrous, p ≥ 97.0%), sodium hydroxide pellets (NaOH, p > 98%), and salicylic acid (purity > 99%) were acquired from Sigma Aldrich. Pure water was produced in the laboratory and used throughout all experiments.

2.2 ZnO NPs synthesis

ZnO powders were obtained by hydrothermal maturation in a microwave field using the synthWAVE equipment (Milestone Srl, Sorisole, Bergamo, Italy). The experimental conditions were chosen based on the best results previously obtained. Briefly, to obtain the ZnO nanostructures further used for the composite materials, the chemical reaction took place in an alkaline environment, starting from $ZnCl_2$ at different concentrations (Table 1). In all cases, the Zn^{2+}: NaOH molar ratio was set to 1:5. The NaOH solution was gradually combined with the $ZnCl_2$ solution under magnetic stirring. The resulting reaction mixtures were transferred into Teflon containers and maintained for 15 min at 100°C, at a pressure of 20 bars, inside an autoclave. At the end of the synthesis, the samples were cooled, the precipitates were separated by centrifugation, washed until a neutral pH, and then dried at 60°C in an oven.

Table 1

Experimental conditions for ZnO NPs synthesis

Sample Name	Zn^{2+} Concentration (mol/L)	Molar Ratio Zn^{2+}:NaOH	Synthesis Method	Reaction Time (min)
ZnO_1	0.1744	1 : 5	Microwave-assisted hydrothermal	15
ZnO_2	0.0872			15
ZnO_3	0.0436			15
ZnO_4	0.0218			15

2.3 Composite wound-dressings synthesis

Sodium alginate was first dissolved at room temperature in pure water to achieve a 2% wt. aqueous solution. For the preparation of the composite materials, each ZnO powder was added into sodium alginate solution (3% wt. of the final dried composite) along with salicylic acid (3% wt. of the final dried composite), sonicated for 1 h, and then magnetically stirred at 25°C for 24 h to remove the air bubbles and ensure good homogeneity. The resulting mixtures were cast into 15 x 100 mm Petri dishes and completely covered by 20 mL of 5% calcium chloride ($CaCl_2$) solution for 5 minutes. This process allows calcium ions to replace sodium ions in the alginate, forming ionic cross-links between the guluronic acid blocks of adjacent alginate chains. After cross-linking, the samples were thoroughly rinsed to remove any excess chloride ions, by immersing the samples in deionized water for 5 min and gently agitating them. The water was replaced 2-3 times until no white precipitate was formed when adding silver nitrate solution. Afterward, the composites were subjected to the freeze-drying process (freezing at -55°C for 12h, vacuum at 0.001 mbar for 12h, and heating under vacuum for 24h to 35°C) to obtain porous composite materials [11]. The resulting materials, with their corresponding composition, are presented in Table 2.

Table 2

Composite wound-dressings composition

Sample Name	ZnO NPs type	Sodium alginate (% wt.)	Salicylic acid (% wt.)
Alg_ZnO_1	ZnO_1	97	-
Alg_ZnO_2	ZnO_2	97	-
Alg_ZnO_3	ZnO_3	97	-
Alg_ZnO_4	ZnO_4	97	-
Alg_ZnO_1_SA	ZnO_1	94	3
Alg_ZnO_2_SA	ZnO_2	94	3
Alg_ZnO_3_SA	ZnO_3	94	3
Alg_ZnO_4_SA	ZnO_4	94	3

2.4 Characterization methods

To investigate the microstructure of the dried composites, a QUANTA INSPECT F50 scanning electron microscope (SEM) (FEI Company, Eindhoven, The Netherlands) equipped with field emission gun—FEG with 1.2 nm resolution and an energy dispersive X-ray spectrometer (EDS) with an MnK resolution of 133 eV was used. Since the analyzed samples lack electrical conductivity, they necessitate a preparation step involving metallization. This process entails coating the surface to be analyzed with a thin film of gold particles approximately 4 nm thick, which takes 60 seconds to complete. Subsequently, the samples are affixed to an aluminum support using a double-sided carbon adhesive tape, which also

conducts electricity. The image acquisition was made on cross-sectioned porous materials, using backscattered electron mode (CBS), as well as on powder samples using secondary electron mode (ETD) [12].

The presence of certain functional groups and the interactions between some components of the composites were investigated by Fourier-transform infrared spectroscopy (FTIR). The investigation consisted of analyzing a small amount of sample using a Nicolet iS50 FTIR spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) in attenuated total reflection mode (ATR), in the wavenumber range 4000 cm^{-1} - 400 cm^{-1}. Each spectrum was obtained by averaging 32 scans, with a resolution of 4 cm^{-1}. The recording of spectral data was possible by connecting the spectrometer to a data acquisition and processing unit through the Omnic program.

The biocompatibility of the synthesized composites was evaluated using XTT reagent (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide) according with the manufacturer protocol (CyQUANT™ XTT Cell Viability Assay Kit, Thermo Fischer Scientific, Waltham, MA, USA). The XTT assay is a colorimetric method used to assess cell viability and proliferation. It measures the metabolic activity of cells, which correlates with the number of viable cells. The assay kit includes the XTT reagent and an Electron Coupling Reagent. The XTT reagent is a tetrazolium-based compound sensitive to cellular redox potential. Actively viable cells convert the water-soluble XTT compound to an orange-colored formazan product. The sensitivity and consistency of the assay is significantly increased when used with the Electron Coupling Reagent. The Human Embryonic Kidney (HEK) cell line was grown in DMEM medium (Sigma-Aldrich, Saint Luis, MO, USA) supplemented with 10% fetal bovine serum, 1% antibiotics (penicillin and streptomycin) (Sigma-Aldrich, Saint Luis, MO, USA), changed twice a week. The cells were placed in 96-well plates, at a density of 3000 cells/well in the presence of Alg-ZnO-SA composites for 24h, and 48 h. The control samples were represented only by cells cultivated in the same condition but without the Alg-ZnO-SA composites. Subsequently, 70 µL of XTT solution was added to the cells, followed by incubation at 37°C for 4 h. After vigorous homogenization of formazan crystals, the absorbance was read at 450 nm using a spectrophotometer.

The cytotoxicity was evaluated using LDH Cytotoxicity Assay Kit (Invitrogen™ CyQUANT™ Thermo Fischer Scientific, Waltham, MA, USA). Quantification of LDH concentration in media is an indicator of cellular cytotoxicity, so the assay can be used to monitor cytotoxicity from the same sample over time. Lactate dehydrogenase (LDH) is a cytosolic enzyme present in many different cell types and the damage of the plasma membrane releases LDH into the surrounding cell culture media. The extracellular LDH in the media can be quantified by a coupled enzymatic reaction in which LDH catalyzes the conversion of lactate to pyruvate via nicotinamide adenine dinucleotide (NAD+) reduction to NADH. Oxidation of NADH by diaphorase leads to the reduction of tetrazolium

salt (INT) to a red formazan product that can be measured spectrophotometrically. The level of formazan formation is directly proportional to the amount of LDH released into the medium, which is indicative of cytotoxicity. To perform the LDH cytotoxicity assay an aliquot of 50 µl cell culture media is transferred to a new plate and 50 µl of LDH reaction mixture is added. After 30 min incubation at room temperature, the assays are stopped by adding the 50 µl of Stop Solution and then fluorescence is measured using a microplate reader at 560 nm excitation and emission of 590 nm.

3. Results and discussion

The morphology and elemental composition of the obtained materials were investigated by SEM and Energy Dispersive X-ray Spectroscopy (EDS) analysis to obtain information about the homogeneity of the materials, as well as the size, distribution, and possible agglomerations of the ZnO particles. In Fig. 1 (a) it can be observed the highly porous nature of Alg_ZnO_3 wound dressing, with well-interconnected pores, which typically results in the freeze-drying process [13].

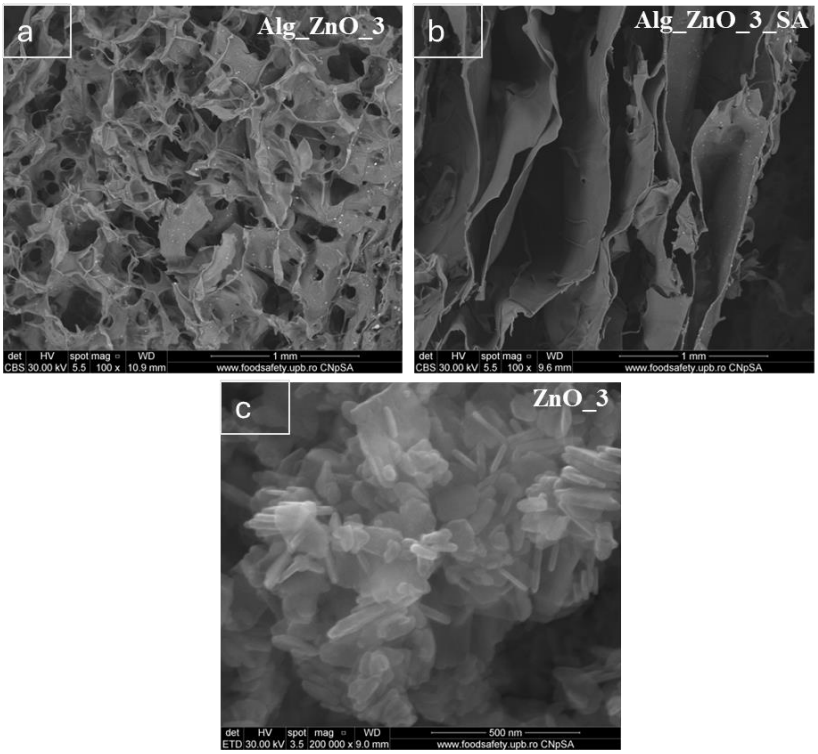

Fig. 1. Cross-section SEM (CBS) images for Alg_ZnO_3 (a) and Alg_ZnO_3_SA (b) composites, along with SEM (ETD) image of ZnO_3 powder (c).

Similar results are obtained for the rest of the samples containing solely alginate and oxide particles. The pores are mostly round-shaped, uniform in size, with diameters ranging between 200 and 500 μm. At low magnification (100x) the ZnO particles seem uniformly dispersed within the alginate matrix. The homogeneous dispersion indicates effective integration of ZnO into the composite, which is crucial for consistent functional properties. The addition of salicylic acid greatly impacts the microstructure of the composites, as it can be seen in Fig. 1 (b). In this case, the previous porous structure, characterized by pores generally spherical and consistent in size, is substituted with layered sheets of polymer, aligned predominantly along the longitudinal axis of the samples, as indicated by the consistent orientation observed in the SEM micrographs.

At higher magnification (Fig. 2), one can better observe the existence of zinc oxide nanoparticles, mainly distributed on the surface of the material, but can also be found inside the alginate pores.

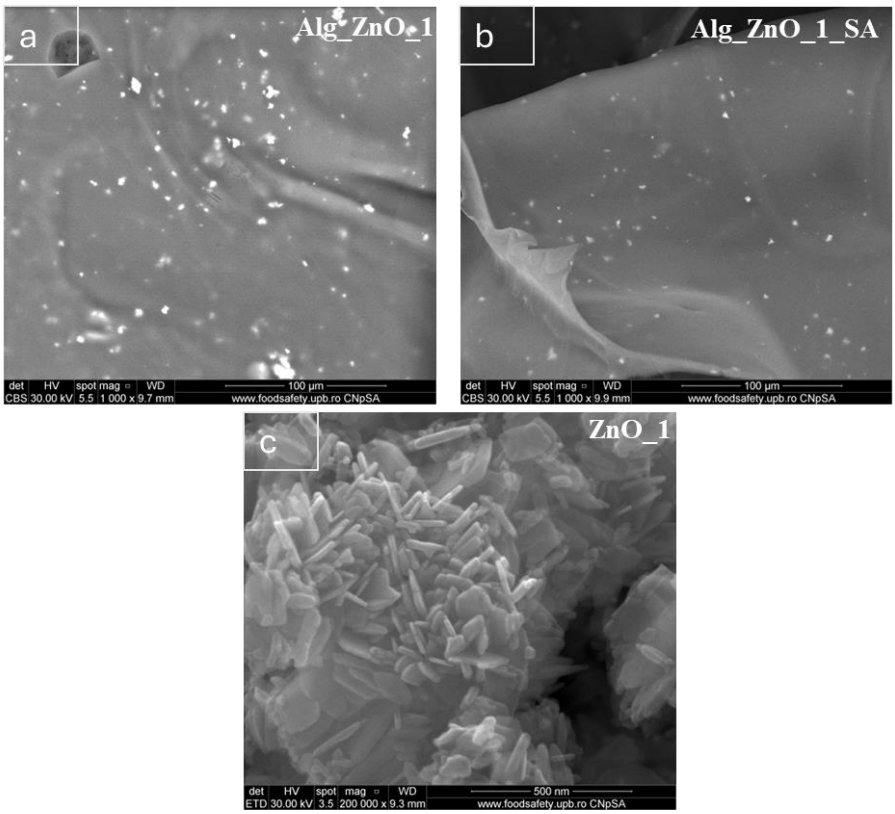

Fig. 2. SEM images (CBS) on the surface of Alg_ZnO_1 (a) and Alg_ZnO_1_SA (b) composites, along with SEM (ETD) image of ZnO_1 powder (c).

The zinc oxide nanoparticles are not perfectly spherical, and, in some areas, they tend to agglomerate, a behavior explained based on polarity and electrostatic

attraction between them. On the other side, it is well-known that zinc oxide nanoparticles are stabilized on the surface of compounds containing functional groups such as alcohols, phenols, amines, and carboxylic acids [14]. Because alginate contains both alcohol and carboxylate groups, the obtained nanoparticles agglomerated on the alginate surface.

EDS analysis was conducted to determine the elemental composition and especially the distribution of Zn in the obtained composites. Fig. 3 illustrates the SEM image on the surface and the associated EDS mapping of Zn in the same area, along with the EDS spectrum for the Alg_ZnO_1 composite sample. The major elements identified are C, O, and Zn, characteristic of the alginate and ZnO particles. Au is also detected, due to the sample preparation method (gold sputtering), while the small contents of chlorine and calcium can be associated with the crosslinking process of alginate. Zn is distributed on the entire analysed area, in some cases forming agglomerates.

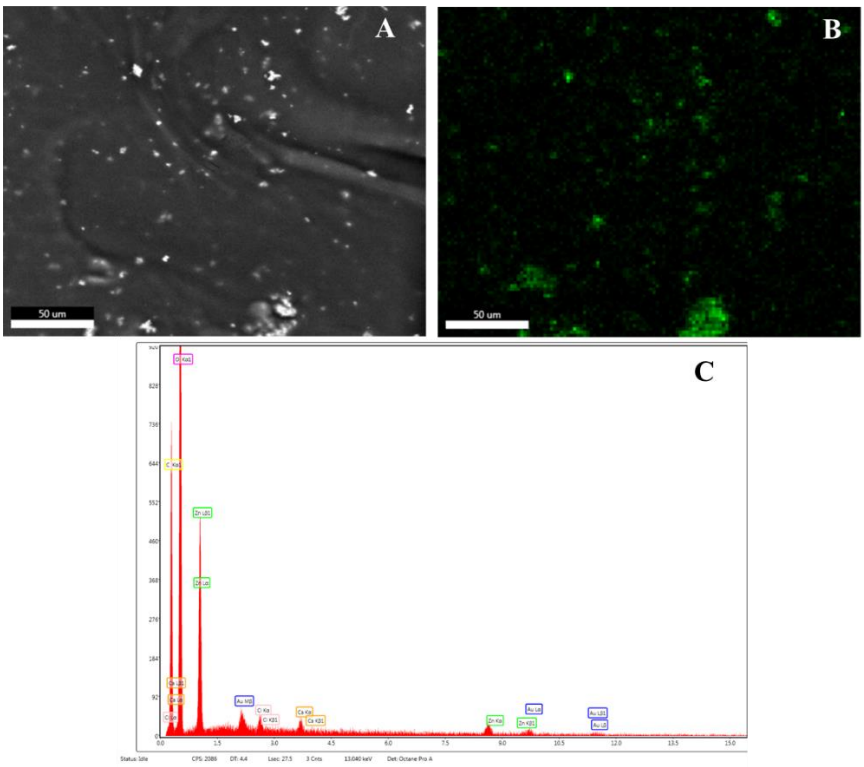

Fig. 3. SEM images on the surface (A), EDS mapping of Zn (B), and EDS spectrum (C) for Alg_ZnO_1 composite

Figs. 4 and 5 present the recorded FTIR spectra of all composites, along with Alg and SA as control samples, to better assess their potential differences in

functional groups and chemical structure. Hence, in all cases, a strong peak is observed around 1600-1650 cm^{-1}, associated with the asymmetric stretching vibrations of the carboxylate groups (COO^-). This is one of the most prominent features in the alginate spectrum [15]. Peaks around 1400-1450 cm^{-1} and 1000-1100 cm^{-1} can be assigned to C-O stretching and C-C stretching vibrations, respectively. These are indicative of the polysaccharide backbone of alginate [16]. A broad absorption band appears around 3200-3600 cm^{-1}, attributed to the O-H stretching vibrations, which is indicative of the hydroxyl groups present in the alginate. Additionally, weaker peaks appear around 2900 cm^{-1} corresponding to C-H stretching vibrations. The presence of the inorganic oxide is evident in the 400-500 cm^{-1} region, where intense peaks corresponding to the Zn-O stretching vibrations appear. The addition of ZnO to the alginate matrix produces changes in most peak's absorbance (or slight shifts to a lower wavenumber), due to interactions between ZnO and the hydroxyl and carboxylate groups of alginate, which can result in hydrogen bonding or coordination bonds [5, 17].

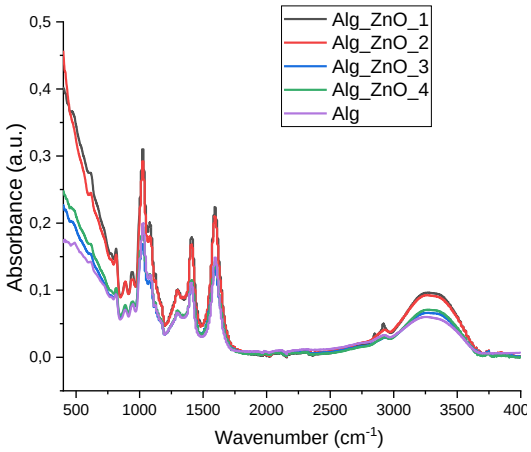

Fig. 4. FTIR spectra of composites containing Alg+ZnO

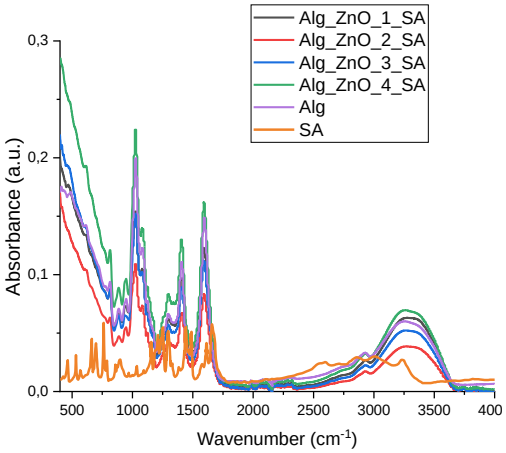

Fig. 5. FTIR spectra of composites containing Alg+ZnO+SA

The biocompatibility of the synthesized composites was evaluated through the XTT assay, using a HEK cell line, and the results after 24 and 48 hours of incubation are presented in Fig. 6. It can be observed that in all cases when the cells were in contact with the obtained composites, the cellular viability was higher than the Control cells. This could be associated with the high biocompatibility of the Alg-ZnO-SA wound dressings. It can be stated that the proposed wound dressings not only have non-cytotoxic effects but even promote cellular proliferation. The cell viability remains high or stable between 24 and 48 hours, indicating sustained biocompatibility of the composites over a longer period. This stability is crucial for potential biomedical applications.

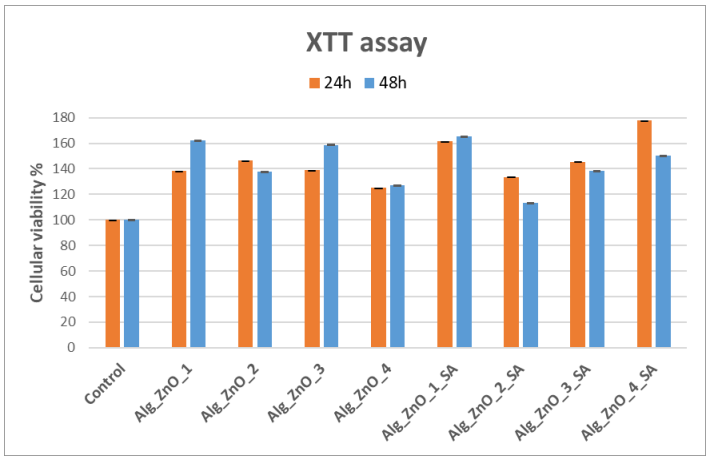

Fig. 6. XTT analysis results after 24h and 48h for the obtained porous samples (presented as mean ± S.D. of 3 replicates)

The HEK cell line is widely used in biomedical research due to its ease of culture and transfection. These cells provide a reliable and consistent model for studying cellular responses to various treatments, including the incorporation of nanoparticles into biomaterials. Moreover, HEK cells are derived from human tissue, making them more representative of human physiology compared to other non-human cell lines. While the antimicrobial activity of the ZnO NPs was extensively studied in the literature [18], ZnO can be cytotoxic at high concentrations. There is a substantial body of literature utilizing HEK cells for nanoparticle toxicity and interaction studies, indicating that cellular viability is affected by ZnO NPs in a dose and time-dependent manner [19-20]. Hence, evaluating the cytotoxicity of the final composites is crucial for prospective medical applications. The cytotoxicity was evaluated using LDH Cytotoxicity Assay and the results are presented in Fig. 7.

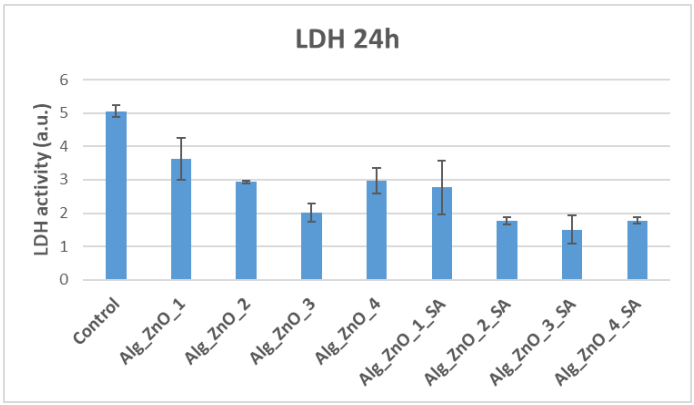

Fig. 7. LDH analysis results after 24h for the obtained porous samples (presented as mean ± S.D. of 3 replicates)

In all cases, the LDH activity observed for the treated cells is lower than that observed for the Control cells, indicating that the Alg-ZnO-SA composites are not causing significant cell membrane damage. All these results suggest that the new composites present low cytotoxicity and good biocompatibility with HEK cells after 24 hours.

4. Conclusions

The development and characterization of sodium alginate-ZnO nanoparticle-reinforced composites incorporating salicylic acid have demonstrated promising results for potential biomedical applications, particularly in advanced wound care solutions. SEM/EDS analysis revealed that the composites exhibit a highly porous structure with well-interconnected pores, crucial for biomedical applications such as wound dressings. The dispersion of ZnO nanoparticles within the alginate matrix appears homogeneous, ensuring consistent functional properties. FTIR analysis highlighted interactions between ZnO and the alginate matrix, particularly through hydrogen bonding and coordination with carboxylate and hydroxyl groups, which are essential for the composite's structural integrity and functional performance. XTT assay demonstrated a high cellular viability of HEK cells in contact with the composites, exceeding that of the control samples. This indicates that the composites are biocompatible and they do not induce significant cytotoxic effects. The sustained cell viability over 48 hours further supports the potential of these materials for prolonged use in biomedical applications. Moreover, the cells contacted with the new composites exhibited lower LDH activity compared to the control cells, indicating minimal cell membrane damage and low cytotoxicity. While these findings are promising, further investigations are required to fully establish their safety and efficacy for applications involving direct contact with human cells.

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