

Development of Biocompatible Ciprofloxacin-Loaded Zinc-Bovine Serum Albumin Nanoflowers as Nontoxic Platform for Local Drug Delivery

Olga Swiech¹, Anna Boguszewska-Czubara², Piotr Golec³, Aleksandra Głowacka³, Marta Prześniak-Welenc⁴, Kamila Sadowska⁵

¹Faculty of Chemistry, University of Warsaw, Pasteura 1, 02-093, Warsaw, Poland; ²Department of Medical Chemistry, Medical University of Lublin, Lublin, Poland; ³Department of Molecular Virology, Institute of Microbiology, Faculty of Biology, University of Warsaw, Warsaw, Poland; ⁴Faculty of Applied Physics and Mathematics, Institute of Nanotechnology and Materials Engineering, Gdansk University of Technology, Gdansk, Poland; ⁵Hybrid and Analytical Microbiosystems Department, Nalecz Institute of Biocybernetics and Biomedical Engineering, Polish Academy of Sciences, Warsaw, Poland

Correspondence: Kamila Sadowska, Hybrid and Analytical Microbiosystems Department, Nalecz Institute of Biocybernetics and Biomedical Engineering, Polish Academy of Sciences, Ks. Trojdena 4, Warsaw, 02-109, Poland, Email ksadowska@ibib.waw.pl

Introduction: Osteomyelitis (OM) is an inflammation of bone and bone marrow and remains one of the most challenging infectious diseases to treat. This clinical difficulty highlights the need for alternative therapeutic strategies, including targeted local delivery of antibacterial agents. In this study, we report for the first time the use of zinc–bovine serum albumin (ZnBSA) hybrid nanoflowers as potential antibiotic carriers for osteomyelitis treatment. Hybrid nanoflowers are hierarchically structured nanomaterials composed of inorganic components (typically metal phosphates) and organic molecules (such as proteins or enzymes) that self-assemble into flower-like morphologies, exhibiting high surface area and synergistic physicochemical properties arising from organic–inorganic integration.

Methods: ZnBSA hybrid nanostructures were synthesized through a rapid and optimized procedure and characterized using scanning electron microscopy, infrared spectroscopy and X-ray diffraction. Ciprofloxacin was loaded into the ZnBSA nanostructures, and drug release behavior was studied by ultraviolet-visible spectroscopy. Antibacterial activity was assessed using optical density measurements and disc diffusion assays. The biocompatibility of ZnBSA loaded with ciprofloxacin was evaluated through in vitro toxicity assays on human red blood cells and dermal fibroblasts, as well as in vivo testing using *Danio rerio*.

Results: The ZnBSA nanostructures demonstrated rapid formation and improved biocompatibility compared to conventional copper-based nanoflowers. Toxicity studies confirmed a favorable safety profile both in vitro and in vivo. Ciprofloxacin-loaded ZnBSA carriers exhibited sustained drug release and effective antibacterial activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Klebsiella pneumoniae*.

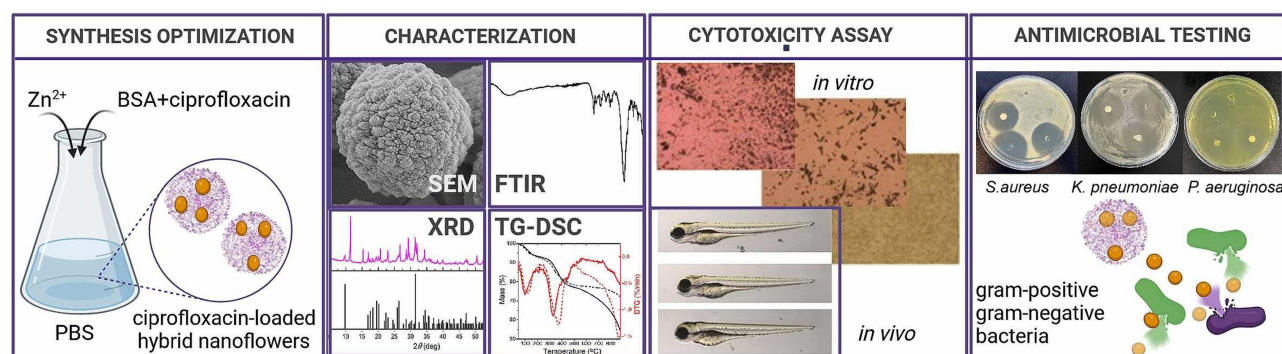
Discussion: These findings indicate that ZnBSA hybrid nanostructures represent a safe and effective localized drug delivery system for osteomyelitis treatment. Their biocompatibility, rapid synthesis, sustained antibiotic release, and broad-spectrum antibacterial activity highlight their potential as a promising alternative to existing nanocarrier systems for managing bone infections.

Keywords: hybrid nanoflowers, drug delivery systems, ciprofloxacin, nontoxic nanostructures

Introduction

Osteomyelitis (OM - from Latin: osteomyelitis) is an inflammation of bone tissue and bone marrow, which is one of the most difficult infectious diseases to cure.¹ Bone infections that are not treated properly can cause serious complications, including osteonecrosis, arthritis, impaired bone growth, and even death. The most frequently isolated pathogens are *Staphylococcus aureus*^{2,3} and *Pseudomonas aeruginosa*,^{4,5} as well as enterococci and streptococci. The primary

Graphical Abstract



challenge in infection treatment today is bacterial resistance to antimicrobial agents, largely driven by inappropriate, insufficient, or excessive use of antibiotics. Alarming, the number of resistant bacterial strains and the breadth of resistance continue to grow. Projections suggest that by 2050, antibiotic resistance could be responsible for up to 10 million deaths annually.⁶ This highlights the urgent need for innovative treatment strategies, such as the localized delivery of antibacterial drugs to infected sites proposed in this study. Local drug delivery focuses on administering the smallest effective dose directly to the affected tissues over a specific time frame. Well-designed local drug delivery systems can minimize off-target side effects, decrease metabolism or clearance rates, lower the frequency of administration, and enhance patient adherence. Local antibiotic delivery has emerged as a promising therapeutic approach to address the limitations of conventional treatments and manage osteomyelitis effectively. The ability to achieve high concentrations of antibiotics within an avascular zone supports its targeted application at the infection site. Furthermore, these elevated concentrations are crucial for eradicating residual organisms within biofilms. Recent achievements in local drug delivery systems were recently summarized in thorough reviews.^{7,8} Due to the type of material from which drug carriers can be synthesized, they can be divided into three categories: (i) organic; (ii) inorganic; and (iii) organic–inorganic (hybrid). Because bones are organic–inorganic (protein–hydroxyapatite) systems, our research focused on the latter category of drug carriers. Recently, we reported the synthesis and characterization of hybrid nanoflowers based on bovine serum albumin (BSA) and hydroxyapatite (HA) for a drug delivery system (DDS) for ciprofloxacin.⁹ Hybrid organic–inorganic flower-like structures were first characterized in 2012 by Ge and colleagues.¹⁰ They reported micrometer-sized protein-copper(II) phosphate(V) structures composed of petal-like formations arranged into hierarchical assemblies, which they named hybrid nanoflowers. Although copper-based nanoflowers are the most studied,^{11,12} they show serious constraints in biomedical applications due to the high toxicity of copper ions. Moreover, Cu-protein nanoflower formation requires a prolonged synthesis time (up to 3 days), which is another limitation of their practical usage.^{10,11} In this context, zinc ions combined with phosphates exhibit a faster reaction rate than copper ions as an inorganic component while also demonstrating no detrimental effects on proteins. In the literature, there are examples of Zn-protein hybrid nanoflowers utilizing lipase,¹³ collagenase,¹⁴ laccase,¹⁵ alcohol dehydrogenase,¹⁶ trypsin¹⁷ or glucose oxidase¹⁸ as an enzymatic component, showing enhanced activity compared to the native form of the enzymes. ZnBSA structures were also reported and tested as adsorbents for heavy metal ions.¹⁹ In this manuscript, we present for the first time Zn-protein nanostructures as drug carriers for the local delivery of antibiotics used in osteomyelitis treatment. The influence of synthesis parameters on ZnBSA nanostructure formation was also studied. Manufactured ZnBSA nanostructures were characterized by Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), scanning electron microscopy (SEM) and thermogravimetry with differential scanning calorimetry (TG-DSC). Toxicity studies *in vitro* (human red blood cells and dermal fibroblasts) and *in vivo* (*Danio rerio* experimental model) were performed. Drug release was evaluated by ultraviolet-visible spectroscopy (UV-vis), and the antibacterial activity against

Pseudomonas aeruginosa, *Staphylococcus aureus* and *Klebsiella pneumoniae* was studied by optical density and disc diffusion methods.

Experimental

Materials

Ciprofloxacin $\geq 98.0\%$ (HPLC) and albumin from bovine serum, minimum 98% electrophoresis; Batch #017K0722 were purchased from Merck (Darmstadt, Germany) and used without further purification. Drug and enzyme solutions were prepared in 10, 50 or 100 mM phosphate-buffered saline (PBS), pH 7.4. PBS buffer was prepared from sodium chloride, NaCl and disodium phosphate, Na_2HPO_4 from POL-AURA (Warsaw, Poland), and potassium chloride, KCl and potassium phosphate monobasic KH_2PO_4 from Merck (Darmstadt, Germany). Water was purified by reverse osmosis in the (RO) Milli-Q station, and its resistivity was $18.2 \text{ M}\Omega\cdot\text{cm}$.

Methods

Nanoflower Synthesis

ZnBSA nanostructures were prepared as follows. In separate flasks, 15, 30, 50 or 75 mg of BSA was dissolved in 10 or 100 mL of 10, 50 or 100 mM PBS, pH 7.4. To each flask containing BSA in PBS, 37 mg of $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$ dissolved in 1 mL of water was added. To check the influence of Zn^{2+} concentration on ZnBSA formation, the experiments were repeated with a doubled amount of $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$ (74 mg). The reaction mixture was kept at room temperature (RT) for a specific time, from 30 min to 96 h. The obtained solids were centrifuged, washed three times with water and left to dry at RT. Samples were denoted XBSAYPBS_Z, where X is the amount of BSA: 15, 30, 50 or 75 mg; Y is the concentration of PBS: 10, 50 or 100 mM; and Z is the PBS volume: 10 or 100 mL.

Preparation of ZnBSA_CF (ZnBSA Nanostructures with Embedded Ciprofloxacin)

Seventy-five milligrams of BSA and 20 mg of ciprofloxacin were dissolved in 100 mL of 10 mM PBS. Next, 37 mg of $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$ dissolved in 1 mL of H_2O was added. Samples were left for 4 hours, centrifuged, washed 3 times with water and left to dry at RT.

Preparation of Gels with ZnBSA

Twenty milligrams of ZnBSA-CF was dispersed in 5 mL of H_2O with 2 mg of ciprofloxacin (ZnBSA-CF sample). To the obtained dispersions, 200 mg of alginate was added and left to completely dissolve (for at least 15 minutes). To the obtained gel, 1 mL of $\text{CaCl}_2\cdot 6\text{H}_2\text{O}$ solution (10 mg/1 mL) was added and mixed well. The prepared gel was poured onto a small petri dish and left to dry at RT. Analogously, pure alginate gel (Alg), alginate gel with ZnBSA sample (Alg-ZnBSA) and alginate gel with CF (Alg-CF) were prepared. All prepared gel variant samples were used in microbiological studies.

Sample Preparation and Release Experiment of Ciprofloxacin from ZnBSA-CF in the Presence of E3 Solution, PBS 7.4 Buffer and Cancer Cell Medium with FBS

Preparation of Samples for the Release Profile in E3 Solution

For the release profile, two initial masses of ZnBSA-CF (0.5 mg and 4.0 mg) were dispersed in 1 mL of E3 solution (prepared as described in Method S1) by vortexing for 3 minutes. The samples were then incubated on a shaker (27°C , 300 rpm) for 236 hours. At predetermined time points (0, 24, 48, 92, 164, and 236 hours), the samples were centrifuged (10 minutes at 6000 rpm). From each supernatant, two aliquots of 20 μL were collected, diluted with E3 solution to a final volume of 1 mL, and analyzed by UV-Vis spectroscopy at $\lambda = 273 \text{ nm}$. Ciprofloxacin concentrations were determined against a calibration curve.

Preparation of Samples for the Release Profile in Cell Medium with FBS

For the release profile, ZnBSA-CF (0.68 mg) was dispersed in 1 mL of cell medium with FBS by vortexing for 3 minutes. As a control, 1 mL of cell medium with FBS without ZnBSA-CF was also prepared. The samples were then incubated on

a shaker (37 °C, 300 rpm) for 340 hours. At predetermined time points (0, 24, 48, 92, 164, 236 and 340 hours), the samples were centrifuged (10 minutes at 6000 rpm). From each supernatant, two aliquots of 20 µL were collected, diluted with water to a final volume of 1 mL, and analyzed by UV–Vis spectroscopy at $\lambda = 273$ nm. The absorbance of the control sample was subtracted from the absorbance of the BSA CF supernatant sample. Ciprofloxacin concentrations were determined against a calibration curve.

Preparation of Samples for the Release Profile PBS Buffer pH 7.4

For the release profile, two samples of ZnBSA-CF (1 mg) were dispersed in 1 mL of PBS buffer (pH 7.4, prepared as described in Method S2) by vortexing for 3 minutes. The samples were incubated on a shaker (37 °C, 300 rpm) for 48 hours. At predetermined time points (0, 2, 20, 24, 44, 48 hours), the samples were centrifuged (10 minutes at 6000 rpm). From each supernatant, two aliquots of 50 µL were collected, diluted with water to a final volume of 1 mL, and analyzed by UV–Vis spectroscopy at $\lambda = 273$ nm. Ciprofloxacin concentrations were determined against a calibration curve prepared in PBS buffer pH 7.4.

Determination of Total CF Concentration

Upon completion of the release profile experiments, 50 µL of the undiluted suspension was taken. Total ciprofloxacin was released by adding 100 µL of 0.2 M HCl and 850 µL of ethanol. After dissolution, the sample was measured by UV–Vis spectroscopy using the calibration curve to determine the final total concentration of ciprofloxacin in the sample.

Physico-Chemical Characterization

Samples were centrifuged using AFI Centrifuge Lisa. Fourier transform infrared (FTIR) spectra were recorded by the ATR method on a Perkin Elmer Frontier spectrophotometer with a resolution of 1 cm^{-1} in the range of 4000 cm^{-1} – 600 cm^{-1} . X-ray diffraction patterns were recorded on an Empyrean 2 Series diffractometer. The measurements were acquired over a 2θ range of 10 – 80° at RT with powdered samples. Scanning electron microscopic imaging was performed using Hitachi TM1000 and ZEISS CrossBeam 540 microscopes. Before scanning, samples were sputtered with gold using Emitech K550X. Thermogravimetric measurements were carried out by using a Netzsch STA 449 F1 thermal analyzer in the temperature range from 40°C to 850°C in an argon atmosphere, with a uniform heating rate of $5^\circ\text{C}/\text{min}$ for all samples. DLS and zeta potential measurements were performed on ZETASIZER ULTRA equipment (Malvern) with ZS XPLORER software. One milligram of ZnBSA or ZnBSA-CF was dissolved in 1 mL of different solutions (PBS buffer pH 7.4, saline, E3 solution, cell medium + FBS or cell medium - FBS). The samples were vortexed for 5 minutes and moved to DLS acrylic cuvettes with four optically active walls. If high polydispersity was detected during the measurement, the samples were diluted 10-fold with the buffer used for the measurement. The following settings were set: cell ZEN0040; material: polystyrene latex; dispersant: water; method builder: Size 3. The measurements were carried out at $T=25^\circ\text{C}$ with 30s of equilibration. Multiple narrow modes were used to process the data. For zeta potential measurements, 800 µL of prepared suspension was moved to the Malvern Panalytical Folded Capillary Zeta Cell. To measure the zeta potential of ZnBSA, the following parameters were set: cell DTS1070; material - polystyrene latex; dispersant - water, method builder - Zeta 3. The measurements were carried out at $T=25^\circ\text{C}$ with 30s of equilibration.

Sample Preparation and Release of Ciprofloxacin from ZnBSA-CF Experiment

Preparation of Samples for the release profile in E3 solution

For the release profile, two initial masses of ZnBSA-CF (0.5 mg and 4.0 mg) were dispersed in 1 mL of E3 solution by vortexing for 3 minutes. The samples were then incubated on a shaker (27°C , 300 rpm) for 236 hours. At predetermined time points (0, 24, 48, 92, 164, and 236 h), the samples were centrifuged (10 minutes, 6000 rpm, RT). From each supernatant, two aliquots of 20 µL were collected, diluted with E3 solution to a final volume of 1 mL, and analyzed by UV–Vis spectroscopy at $\lambda = 273$ nm. Ciprofloxacin concentrations were determined against a calibration curve.

Preparation of Samples for the Release Profile in Cell Medium with FBS

For the release profile, ZnBSA-CF material (0.68 mg) was dispersed in 1 mL of cell medium with FBS by vortexing for

3 minutes. As a control, 1 mL of cell medium with FBS without ZnBSA-CF was also prepared. The samples were then incubated on a shaker (37 °C, 300 rpm) for 340 hours. At predetermined time points (0, 24, 48, 92, 164, 236 and 340 h), the samples were centrifuged (10 minutes, 6000 rpm, RT). From each supernatant, two aliquots of 20 µL were collected, diluted with water to a final volume of 1 mL, and analyzed by UV–Vis spectroscopy at $\lambda = 273$ nm. The absorbance of the control sample was subtracted from the absorbance of the BSA CF supernatant sample. Ciprofloxacin concentrations were determined against a calibration curve.

Preparation of Samples for the Release Profile in PBS Buffer pH 7.4

For the release profile, two samples of ZnBSA-CF (1 mg) were dispersed in 1 mL of PBS buffer (pH 7.4) by vortexing for 3 minutes. The samples were incubated on a shaker (37 °C, 300 rpm) for 48 hours. At predetermined time points (0, 2, 20, 24, 44, 48 h), the samples were centrifuged (10 minutes, 6000 rpm, RT). From each supernatant, two aliquots of 50 µL were collected, diluted with water to a final volume of 1 mL, and analyzed by UV–vis spectroscopy at $\lambda = 273$ nm. Ciprofloxacin concentrations were determined against a calibration curve prepared in PBS buffer pH 7.4.

Determination of the Total CF Concentration in ZnBSA-CF

Upon completion of the release profile experiments, 50 µL of the undiluted ZnBSA-CF suspension was taken. Total ciprofloxacin was released by adding 100 µL of 0.2 M HCl and 850 µL of ethanol. After dissolution, the sample was measured by UV–Vis spectroscopy using the calibration curve to determine the final total concentration of ciprofloxacin in the sample.

Toxicity in the Danio Rerio experimental Model in vivo

To determine the toxicity of ZnBSA, ZnBSA-CF and CF, a modified version of the fish embryo toxicity (FET) test was performed on zebrafish (*Danio rerio*) according to OECD Test Guideline 236.²⁰ Briefly, newly fertilized zebrafish eggs were placed in 24-well plate with 5 embryos per well in 1 mL of E3 solution. Then, appropriate amounts of ZnBSA (0.25–1 mg), ZnBSA-CF and corresponding amounts of CF alone (calculated from release profile: 0.078–0.312 µg/mL) were added to wells and incubated for 96 h at 28 ± 0.5 °C under a light/dark period of 12/12 h. At the end of the exposure period (96 hpf, hours post-fertilization), acute toxicity was determined based on a positive outcome in any of the four visual indicators of lethality, including coagulation of fertilized eggs, lack of somite formation, lack of detachment of the tailbud from the yolk sac and lack of heartbeat.

Hemolytic Activity

Mouse blood was used for experiments, with no procedures performed on live animals. Samples were collected in sterile tubes containing heparin as an anticoagulant. To isolate erythrocytes, the blood was centrifuged at $500 \times g$ for 10 minutes at 4 °C, after which the plasma (supernatant) was discarded. The erythrocyte pellet was then resuspended in PBS buffer and centrifuged again under the same conditions. This washing step was repeated several times until the supernatant became clear. Following the final wash, erythrocytes were resuspended in PBS to achieve a final concentration of 2%. Subsequently, 1 mL of this 2% erythrocyte suspension was mixed with appropriate amounts of ZnBSA (0.25–1 mg), ZnBSA-CF or free CF (0.078–0.312 µg/mL, based on release profile) and incubated at 37 °C for 24 hours. After incubation, the samples were centrifuged at $5000 \times g$ for 10 minutes, and absorbance was recorded at 415 nm. As a positive control, a 1% Triton X-100 solution was used to induce complete hemolysis (100%). The morphology of erythrocytes was observed under a microscope, and images were taken. The ratio of erythrocytes to echinocytes was calculated after image analysis.

Cytotoxicity Assay

To evaluate the cytotoxic effect of nanoflowers, human skin fibroblasts (BJ cell line: ATCC-CRL-2522™) were used. First, the toxicity of CF was evaluated in BJ cells. For the treatment, the cells were seeded in 96-well plates in complete EMEM culture medium and left in the incubator for 24 hours for attachment. Then, appropriate concentrations of CF (0–600 µg/mL) were dissolved in serum-free EMEM medium and incubated for 24, 48 and 72 hours. After

that time, the MTT test was performed to evaluate the percentage of cell viability. The absorbance was measured using a SYNERGY H2 plate reader, and the results are presented as a dose response curve with evaluated values of IC₅₀. Then, the cells were seeded in 24-well plates in complete EMEM culture medium and left in the incubator for 24 hours for attachment. Then, appropriate amounts of ZnBSA (0.25–1 mg), ZnBSA-CF or free CF (0.078–0.312 µg/mL, based on the release profile) were added to 1 mL of serum-free EMEM and incubated for 72 hours. After that time, images of the cells were taken, and the MTT test was performed to evaluate cell viability. The results are presented as bars of cell viability.

Macrophage Cytotoxicity Assay

To assess the cytotoxicity of nanoflower formulations, murine macrophage-like cells (RAW 264.7; ATCC® TIB-71™) were used. Initially, the toxicity of free CF was examined. RAW 264.7 cells were seeded in 96-well plates in complete DMEM and allowed to adhere for 24 hours under standard culture conditions (37 °C, 5% CO₂). After this period, the medium was replaced with serum-free DMEM containing various concentrations of CF (0–600 µg/mL). The cells were then incubated for 24, 48, and 72 hours. At each time point, cell viability was determined using the MTT assay. Absorbance was measured using a SYNERGY H2 microplate reader, and the results were expressed as dose–response curves with corresponding IC₅₀ values.

In a separate experiment, RAW 264.7 cells were seeded in 24-well plates and allowed to adhere for 24 hours. Then, different concentrations of ZnBSA (0.25–1 mg), ZnBSA-CF, or free CF (0.078–0.312 µg/mL, based on the release profile) were added to 1 mL of serum-free DMEM. After 72 hours of incubation, microscopy images of the cells were captured to assess morphological changes. The MTT assay was subsequently performed to quantify cell viability, and the results are presented as bar graphs representing the percentage of viable cells.

Antibacterial Activity Assays

To assess the antibacterial activity of ZnBSA-CF nanoflowers, pathogenic strains of *S. aureus*, *P. aeruginosa*, and *K. pneumoniae* were used. The strains were obtained from the Department of Molecular Virology's collection at the University of Warsaw and were originally isolated from canine infections. Species identification was carried out using the MALDI Biotyper® system (Bruker) based on matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Antibiotic susceptibility profiles, including ciprofloxacin resistance, were verified using the VITEK-2 system (bioMérieux). All bacterial strains were cultured under standard laboratory conditions on LB agar plates or in LB broth at 37 °C. All experiments were performed in triplicate.

For the agar disk diffusion assay, 100 µL of overnight bacterial cultures were spread on LB agar plates. For the surface, either blank diffusion disks (5 mm diameter, BIOMAXIMA) soaked with 25 µL of CF solution (0.2 mg mL⁻¹), corresponding to a total amount of 5 µg of ciprofloxacin per disk (in line with the EUCAST reference dose for susceptibility testing), or 5 mm fragments of hydrogel samples (Alg, Alg-ZnBSA, Alg-CF or ZnBSA-CF), were placed. The plates were incubated at 37 °C for 16 h, after which the diameters of the bacterial growth inhibition zones were measured and analyzed.

For bacterial colony counting and optical density measurements, the same overnight cultures were used. A total of 1 mL of bacterial suspension was inoculated into 100 mL of fresh LB medium and incubated with shaking at 37 °C until the culture reached an OD₆₀₀ of ~0.2. The culture was then divided into five flasks (20 mL each), and a 100 µL aliquot was taken for baseline colony-forming unit (CFU) analysis. Gel fragments: Alg, Alg-ZnBSA, or ZnBSA-CF, cut to match the dimensions of 5 mm disks, were added to the appropriate flasks. A flask with no additive served as an untreated control. As a control for the effect of CF, a culture with the addition of 25 µL of ciprofloxacin at a concentration of 0.2 mg/µL was used. Cultures were incubated with shaking at 37 °C. At 2 and 4 h posttreatment, 1 mL samples were collected for OD₆₀₀ measurement, and 100 µL was withdrawn for CFU analysis. Optical density was measured using a cuvette-based spectrophotometer (BioSpectrometer, Eppendorf). For CFU enumeration, serial dilutions were prepared in PBS, and 100 µL of each dilution was plated on LB agar. After incubation for 16 h at 37 °C, colonies were counted, and CFU mL⁻¹ values were calculated.

Results

Synthesis and Characterization of ZnBSA Nanostructures

Although metal-protein nanoflowers have been known for years, the synthesis of Zn-based nanoflowers has not been studied in detail. Therefore, in the first step, we studied the parameters of synthesis, such as time, concentration and volume of PBS and the ratio of reagents. The obtained materials were characterized by SEM, FTIR and XRD. The exemplary SEM images, FTIR spectra and XRD patterns of samples synthesized in 100 mL of 10 mM PBS with different BSA concentrations are presented in [Figure 1A–D](#). SEM images and FTIR spectra of other samples are shown in [Figure S1–S4](#) in the [Supplementary file](#). The presence of protein in the obtained structures was confirmed by FTIR spectroscopy. In the case of samples prepared in 10 mM 100 mL PBS, bands at 1638 and 1521 cm^{-1} referring to BSA were clearly visible, and no distinct differences among samples were revealed in the FTIR spectra (see [Figure 1F](#)). However, if the concentration of buffer solution was higher (50 or 100 mM), the FTIR spectra showed a substantial decrease in BSA bands, and the spectra revealed mainly bands characteristic of phosphates in the range of 110–930 cm^{-1} and 630–540 cm^{-1} and water of crystallization at 1640 cm^{-1} (see [Figure S2](#)). Similarly, elongation of time increased the content of the inorganic component in the sample, which was proven by the FTIR spectra and SEM images presented in [Figure S1](#). The inorganic phase in the ZnBSA samples was characterized by XRD analysis, and the selected diffractograms are shown in [Figure 1E](#). The presented diffractograms obtained for samples 15BSA10PBS_100, 30BSA10PBS_100 and 50BSA10PBS_100 coincide with the orthorhombic $\text{Zn}_3(\text{PO}_4)_2 \cdot 4\text{H}_2\text{O}$ (β -hopeite) XRD pattern (JCPDS 00–033-1474). In the case of the sample with the highest BSA content, namely, 75BSA10PBS_100, the inorganic phase is monoclinic zinc phosphate monohydrate ($\text{Zn}_3(\text{PO}_4)_2 \cdot \text{H}_2\text{O}$; JCPDS 01–077-5374). The control sample, obtained without BSA, was a mixture of hydrated zinc phosphates.^{21,22} The decrease in the hydration degree of zinc phosphate in the ZnBSA nanostructures observed for the samples obtained with the highest BSA concentration in the reaction mixture is in agreement with the already proposed mechanism of hybrid nanoflower formation. Briefly, in PBS medium, positively charged metal ions and negatively charged phosphates react to form metal phosphate nanocrystals (in the majority of reports, these are hydrates of metal phosphates). Organic molecules, such as proteins, form complexes with metal ions through coordination. If the number of accessible protein molecules increases, more coordination bonds can be created with metal ions, superseding water molecules and taking part in complex formation. As a result, lower hydrated zinc phosphate is formed ($\text{Zn}_3(\text{PO}_4)_2 \cdot \text{H}_2\text{O}$), with smaller crystallites compared to the samples obtained in the reaction with a lower protein-to-metal ratio. Analyzing the results, the synthesis protocol, in which 37 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ in 1 mL of water was added to 75 mg of BSA dissolved in 100 mL of 10 mM PBS and kept for 4 h, was selected for further studies. These samples were nearly spherical ZnBSA nanoflowers (see [Figure 1D](#) and [Figure S5](#)), with zinc phosphate crystals in the range of 27 ± 1 nm, as confirmed by XRD and SEM analysis and higher BSA content (as compared to other samples), as proven by FTIR studies. Ciprofloxacin, an antibiotic used to treat osteomyelitis, was incorporated into ZnBSA nanostructures as detailed in the Experimental section to produce the ZnBSA-CF sample.

The successful embedding of CF into the ZnBSA delivery system was proven by FTIR (see [Figure S6](#)) and TG-DSC (thermogravimetry-differential scanning calorimetry) analyses. TG and DSC curves are shown in [Figure 2A](#) and [B](#).

In the temperature range of 40–350°C, two steps of mass loss are present in the ZnBSA sample, referring to the dehydration of zinc phosphate and denaturation of BSA.^{23,24} These endothermic processes appear in the DSC curve shown in [Figure 2B](#). Further increasing the temperature led to the decomposition of the sample, which is an exothermic phenomenon reflected in the DSC curve of the ZnBSA sample. Above 750°C, recrystallization of the zinc phosphate is observed. Similar behavior is detected for the ZnBSA-CF sample up to 300°C, but with an additional step between 350–450°C coinciding with CF degradation.²⁵ The total mass loss of the ZnBSA-CF sample was higher than that observed for ZnBSA (residual mass 51% and 71% of the initial mass, respectively), which can be attributed to the presence of approximately 20% CF in the sample.

Hydrodynamic size measurements confirmed that the unloaded ZnBSA nanoflowers exhibited submicron-scale colloidal assemblies with Z-average diameters ranging from 590 nm to 1015 nm depending on the dispersion medium ([Table S1](#)). The smallest sizes were observed in cell culture medium supplemented with FBS (592 ± 61 nm), suggesting partial stabilization by serum proteins, while the largest were recorded in PBS (1015 ± 100 nm). PDI values varied

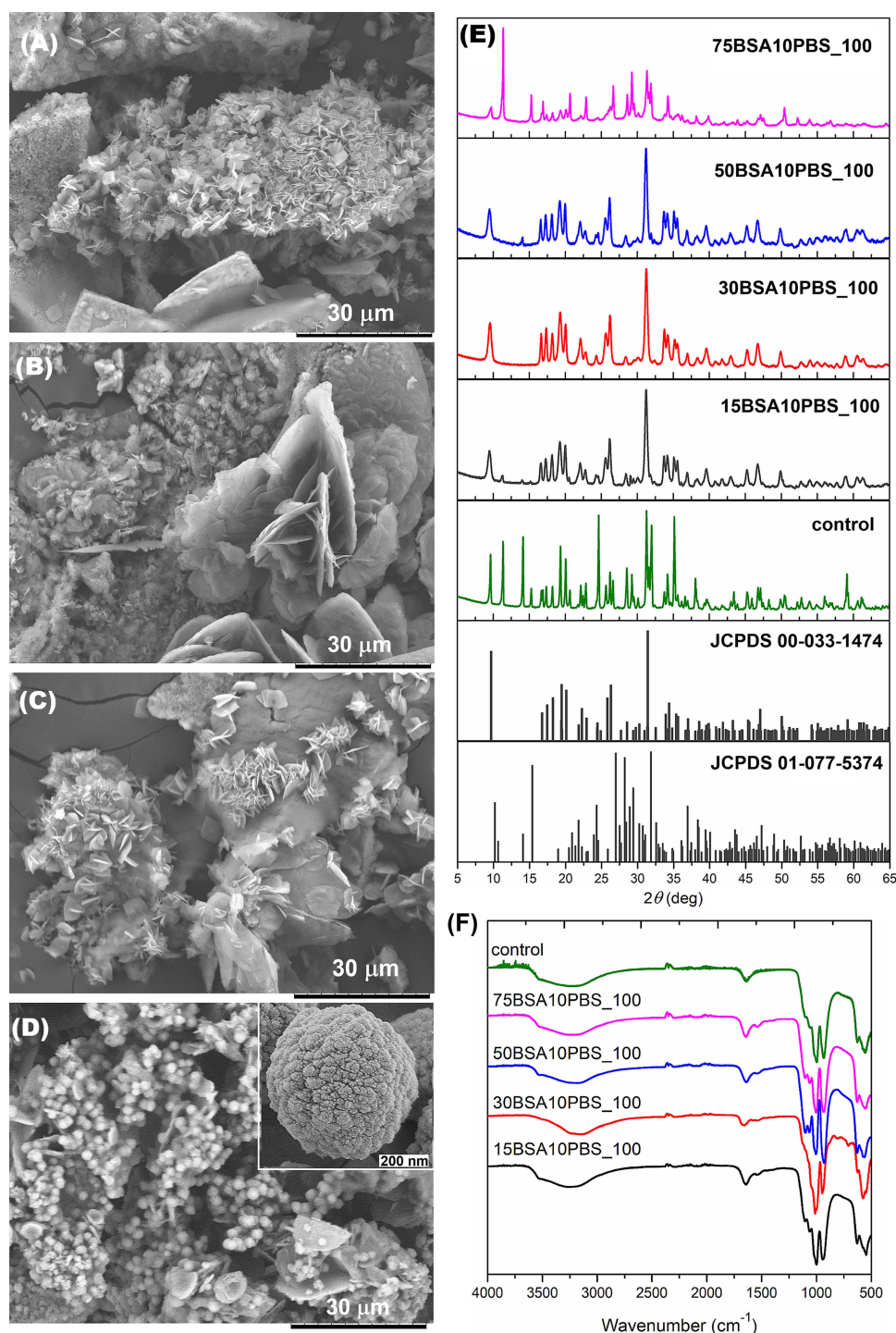


Figure 1 SEM images of ZnBSA nanostructures synthesized in 100 mL of 10 mM PBS, pH 7.4 with different amounts of BSA labeled (A) 15BSA10PBS_100. (B) 30BSA10PBS_100. (C) 50BSA10PBS_100 and (D) 75BSA10PBS_100. XRD (E) and FTIR (F) results of ZnBSA nanostructures synthesized in 100 mL of 10 mM PBS, pH 7.4 with different amounts of BSA.

between 0.29 and 0.62, indicating heterogeneous but acceptable distributions in line with the complex flower-like morphology. The surface charge of the unloaded nanostructures was moderately negative (−11.7 to −18.1 mV), consistent with the presence of BSA and phosphate groups.

Loading CF into ZnBSA resulted in a pronounced increase in hydrodynamic size across all media, with Z-average values from 1530 nm in PBS to over 2500 nm in E3 solution and up to ~2800 nm in saline. Despite this increase, the PDI

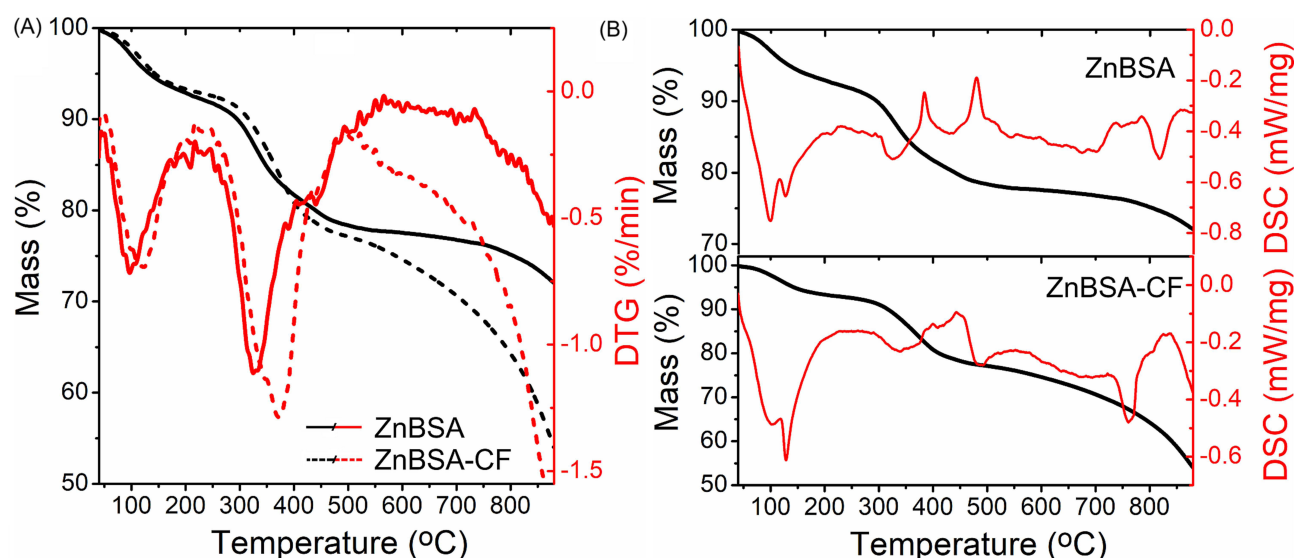


Figure 2 (A) TG and DTG curves of ZnBSA and ZnBSA-CF. (B) TG and DSC curves of ZnBSA and ZnBSA-CF at a heating rate of 10°/min under Ar.

values for ZnBSA-CF (0.23–0.64) remained within the range observed for unloaded nanoflowers. Importantly, ciprofloxacin loading did not markedly alter the surface charge, with zeta potentials remaining in the range of -14 to -18 mV. Notably, in serum-containing medium, the negative charge was stable over time ($T_0 = -18.15$ mV, $T_{8h} = -16.38$ mV), indicating that protein corona formation did not destabilize the formulation.

Toxicity Studies of ZnBSA and ZnBSA-CF Nanoflowers

The aim of this study was to develop a hybrid material that mimics bone structure to function as a delivery system for ciprofloxacin, an antibiotic commonly used to treat osteomyelitis. To ensure the safety and biocompatibility of the proposed system, comprehensive toxicity studies were conducted on both ZnBSA and ZnBSA-CF samples. This work represents the first report presenting both *in vitro* and *in vivo* toxicity assessments of organic–inorganic nanoflowers.

Danio Rerio Experimental *in vivo* Model

These evaluations are crucial for understanding the potential biological risks associated with the material and for validating its suitability for clinical applications. In the conducted *in vivo* experiments using zebrafish embryos, neither ZnBSA nor ZnBSA-CF nanoflower formulations exhibited observable toxicity. No mortality or adverse developmental outcomes were detected following treatment with these materials. In contrast, free ciprofloxacin (CF) demonstrated a dose-dependent toxic effect. Toxicity was observed at concentrations corresponding to the release-equivalent dose of 0.75 mg of nanoflower formulation, which, based on the release profile, equals approximately 0.234 $\mu\text{g/mL}$ of CF. At this concentration and above, 100% mortality of zebrafish embryos was recorded (Figure 3). The observed toxicity threshold for free CF is consistent with the solubility-limited release profile obtained in E3 solution. The raw UV–Vis spectra and drug release profile are presented in Figure S7 and Figure S8, respectively. At the lower ZnBSA-CF initial concentration (0.5 mg ZnBSA-CF [0.15 mg CF]/mL), 42% of the CF (0.06 mg/mL) was released after 92 h. In contrast, at higher initial concentration (4.0 mg ZnBSA-CF [1.26 mg CF]/mL), release reached only 10% (0.15 mg CF/mL) before reaching a plateau due to solubility limitations. Therefore, regardless of the initial vehicle concentration, the effective CF concentration in E3 was limited to approximately 0.15 mg/mL. Thus, regardless of the initial vehicle concentration, the effective CF concentration in E3 was limited to ~ 0.15 mg/mL. This value is below the free CF concentration level at which 100% mortality of zebrafish embryos was observed (Figure 3).

Importantly, no developmental malformations (eg, body shape abnormalities, pericardial edema) or disturbances in heart rate were observed in embryos treated with ZnBSA or ZnBSA-CF, suggesting a favorable safety profile of the tested nanoflower formulations. The toxic effects observed in the group treated with free CF can therefore be attributed to

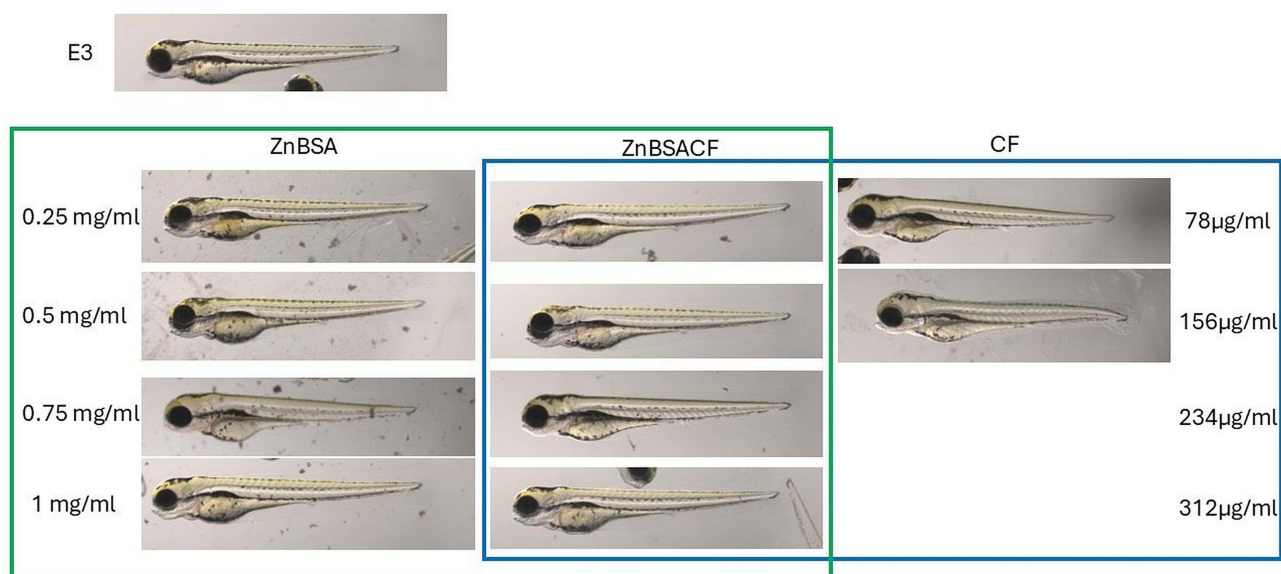


Figure 3 Ecotoxicity of nanoflower formulations in the *Danio rerio* experimental model.

the drug itself rather than the delivery system. This toxicity is likely associated with the acidification of the surrounding medium, as ciprofloxacin was used in the form of its hydrochloride salt ($\text{CF} \cdot \text{HCl}$). Increasing doses of CF led to a decrease in pH, which in turn negatively affected embryo viability.

Interestingly, such toxic effects were not observed for ZnBSA-CF nanoflowers containing the same total amount of CF. This indicates that the controlled and gradual release of the drug from the nanoflowers significantly reduces acute toxicity. This sustained release profile could be advantageous in clinical applications, for example, as a localized antibiotic delivery system in bone implants, offering effective antimicrobial protection during tissue regeneration without the need for systemic antibiotic therapy, which often burdens the entire organism and may contribute to adverse effects or resistance development.

Hemolytic Activity

Evaluation of red blood cells (erythrocytes) is a critical step in determining the safety of materials and chemical compounds intended for internal use, especially those that come into direct contact with blood, such as drug delivery systems, biomedical implants, or intravenous therapeutics. Due to their sensitivity to physicochemical changes in their environment, erythrocytes (Figure 4A) serve as a reliable model for assessing cytotoxicity and biocompatibility. Two commonly used methods include spectrophotometric hemolysis assays and morphological examination of erythrocyte integrity and deformation. Echinocytes (Figure 4B), also known as burr cells, are erythrocytes that exhibit a spiky or crenated appearance due to changes in the plasma membrane. Their formation can be triggered by various factors, including pH alterations, osmotic stress, chemical exposure or prolonged storage. The presence of echinocytes is generally considered a marker of sublethal stress or early cytotoxicity.²⁶ Therefore, monitoring the ratio of normal erythrocytes to echinocytes provides insight into the impact of the tested materials on red blood cell morphology (Figure 4C). A higher erythrocyte-to-echinocyte ratio indicates a lower degree of erythrocyte deformation and thus better biocompatibility. Ratios above 1 reflect a predominance of healthy, biconcave-shaped erythrocytes, while values below 1 indicate increasing morphological stress. In our study (Figure 4D, Figure S9), ZnBSA nanoflowers maintained a ratio close to or above 1, even at the highest tested concentration (1 mg). Notably, at 0.25 mg, ZnBSA showed a better erythrocyte profile than the untreated control group. The ZnBSA-CF formulation (ciprofloxacin-loaded nanoflowers) at 0.25 mg behaved similarly to ZnBSA, also demonstrating a ratio higher than that of the control. However, increasing the concentration of ZnBSA-CF led to a gradual decrease in the erythrocyte/echinocyte ratio, approaching the values observed for free CF, which reached approximately 0.7 at the highest dose. In comparison, the lowest value for

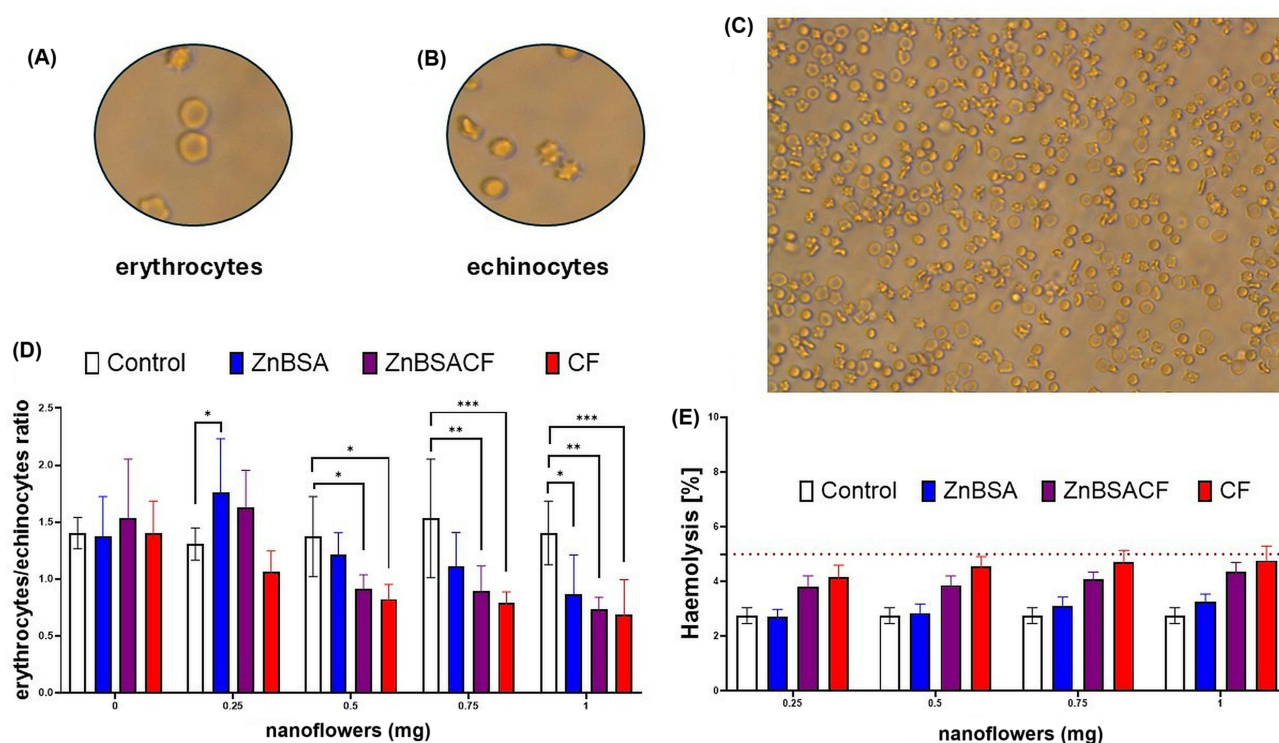


Figure 4 Effects of nanoflower formulations on the morphological quality of erythrocytes. (A) Image of erythrocytes. (B) Image of echinocytes. (C) Image of erythrocytes after 72 h incubation with ZnBSA. (D) Ratio of normal erythrocytes to echinocytes after nanoflower exposure. (E) Effect on hemolysis. The amount of nanoflowers is expressed in milligrams, while ciprofloxacin (CF) loading is also reported relative to the nanoflower mass, taking into account that CF constitutes 30% (w/w), ie, for every 1 mg of nanoflowers, 0.312 μ g of CF is incorporated. Data are presented as the mean $\pm$ SD ($n = 6$); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared to control.

ZnBSA-CF was 0.8 at the highest dose applied (1 mg), suggesting that nanoencapsulation improved the erythrocyte compatibility of ciprofloxacin. These results may be partially explained by the acidifying effect of ciprofloxacin hydrochloride, which at higher concentrations lowers the pH of the surrounding medium and contributes to red blood cell stress. Notably, ciprofloxacin itself does not exhibit hemolytic properties, so the observed morphological changes likely stem from indirect effects such as acidification. Additionally, echinocyte presence in the control group may be attributed to the type of anticoagulant used (heparin) and prolonged incubation time, as morphological assessment was performed after 72 hours, much longer than standard exposure periods.²⁷

The hemolysis results are consistent with the release profile obtained in PBS buffer (pH 7.4, [Figure S10](#)). For the ZnBSA-CF solution in PBS (CF concentration 0.096 mg/mL), approximately 40% of the total drug dose (~ 0.04 mg/mL) was released within 24 h, after which the curve reached a plateau without reaching full release. Despite this relatively rapid release, hemolysis remained below 5% for all doses tested ([Figure 4E](#)).

Quantitative hemolysis was also assessed spectrophotometrically by measuring the release of hemoglobin compared to a positive control (100% hemolysis) induced by 1% Triton X-100, which causes complete disruption of erythrocyte membranes. Values in the range of 0–5% are generally accepted as safe, while hemolysis exceeding 5–10% may indicate weak compatibility. The results exceeding 10% are often considered cytotoxic and require further investigation. In our experiment ([Figure 4E](#)), ZnBSA samples yielded absorbance values comparable to those of the control group, indicating negligible hemolysis. For ZnBSA-CF and free CF, hemolysis levels increased with dose; however, even at the highest concentrations, they did not exceed 5% hemolysis. In toxicological terms, hemolysis values below 5% are considered very low and acceptable, especially given our extended incubation time of 24 hours, whereas standard hemolysis protocols typically use only 1 hour of exposure.²⁸ For reference, the control group in our study showed 2.7% hemolysis, likely reflecting baseline stress due to prolonged incubation or sample handling.

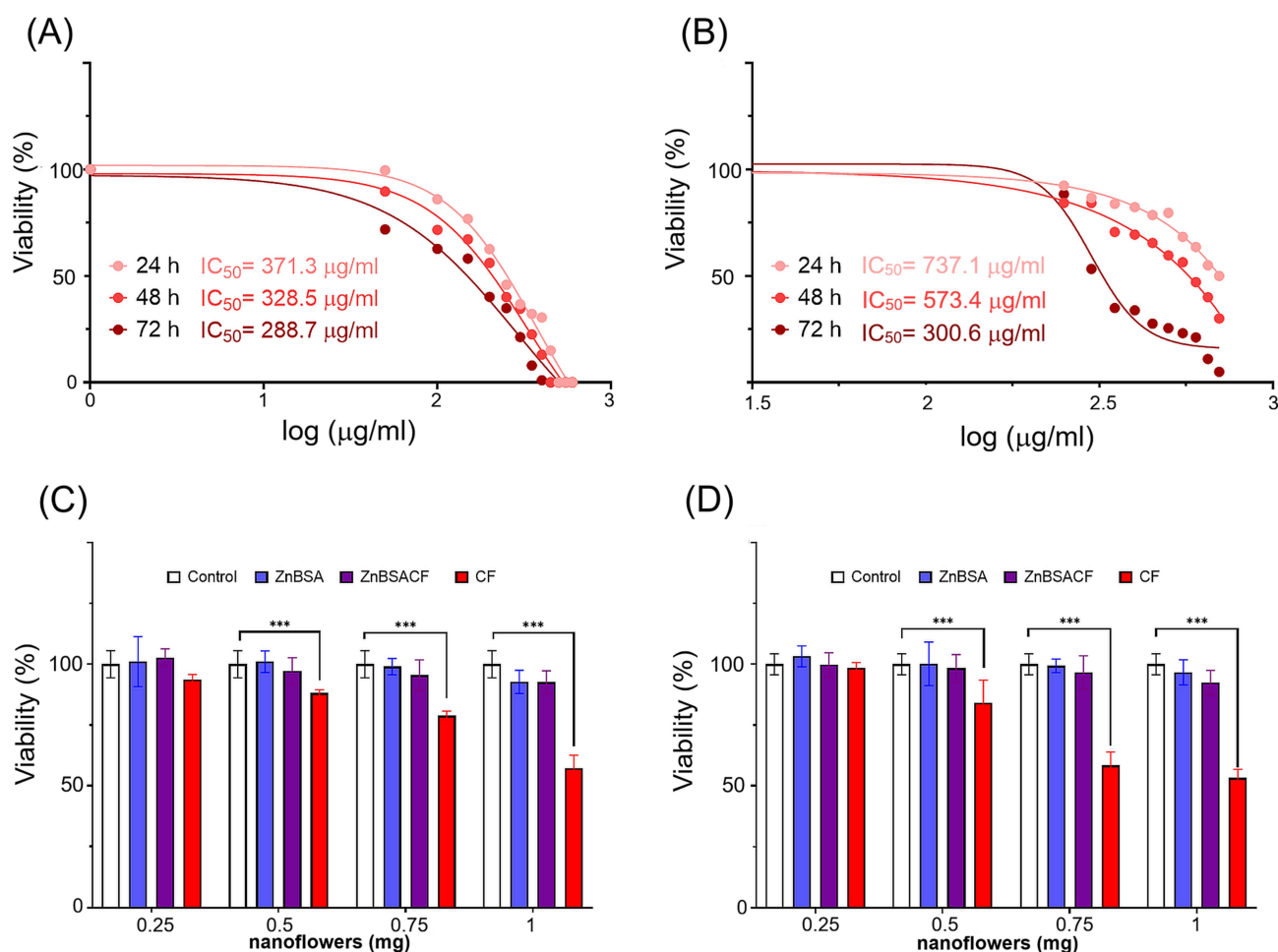


Figure 5 Evaluation of the cytotoxicity of ciprofloxacin hydrochloride (CF-HCl), ZnBSA, and ZnBSA-CF formulations in human dermal fibroblasts (BJ) and murine macrophages (RAW 264.7). **(A)** Dose-response curves for CF-HCl for BJ and **(B)** RAW 264.7 cells after 24, 48, and 72 hours of incubation. IC₅₀ values decrease over time, indicating time-dependent cytotoxicity. **(C)** Cell viability of BJ and **(D)** RAW 264.7 cells after 72 hours of exposure to ZnBSA, ZnBSA-CF or CF at equivalent concentrations based on release profile. The amount of nanoflowers is expressed in milligrams, while ciprofloxacin (CF) loading is also reported relative to the nanoflower mass, taking into account that CF constitutes 30% (w/w), ie, for every 1 mg of nanoflowers, 0.312 μg of CF is incorporated. Data are presented as the mean ± SD (n = 6); ***p < 0.001 compared to the control.

Cytotoxicity Assay

The cytotoxic potential of ZnBSA and ZnBSA-CF nanoformulations was evaluated using human dermal fibroblasts (BJ cell line) through an MTT assay. The results demonstrated that both ZnBSA and ZnBSA-CF exhibited excellent cytocompatibility, with cell viability consistently remaining above 92% even at the highest tested dose (Figure 5B). These findings suggest that neither the unloaded nor the ciprofloxacin-loaded nanoflowers negatively affected fibroblast proliferation over 72 hours of exposure. In contrast, free ciprofloxacin (CF) showed a dose-dependent decrease in cell viability, with viability values dropping to approximately 93%, 87%, 78% and 57% for increasing concentrations (Figure S11). These results correlate well with the IC₅₀ value of 288 μg/mL determined after 72 hours of incubation (Figure 5A), indicating a moderate cytotoxic effect of the free drug on fibroblasts.

Macrophage Cytotoxicity Assay

Cytotoxicity testing in RAW 264.7 murine macrophages revealed a high level of biocompatibility for both ZnBSA and ZnBSA-CF nanoformulations, with cell viability consistently exceeding 90% even at the highest tested concentrations after 72 hours (Figure 5D, Figure S12). These results align closely with those observed for human fibroblasts (BJ cells), confirming the overall safety of the nanoflowers in both structural and immune cell models. In contrast, ciprofloxacin hydrochloride (CF-HCl) exhibited significant cytotoxicity in a dose-dependent manner, with cell viability decreasing to

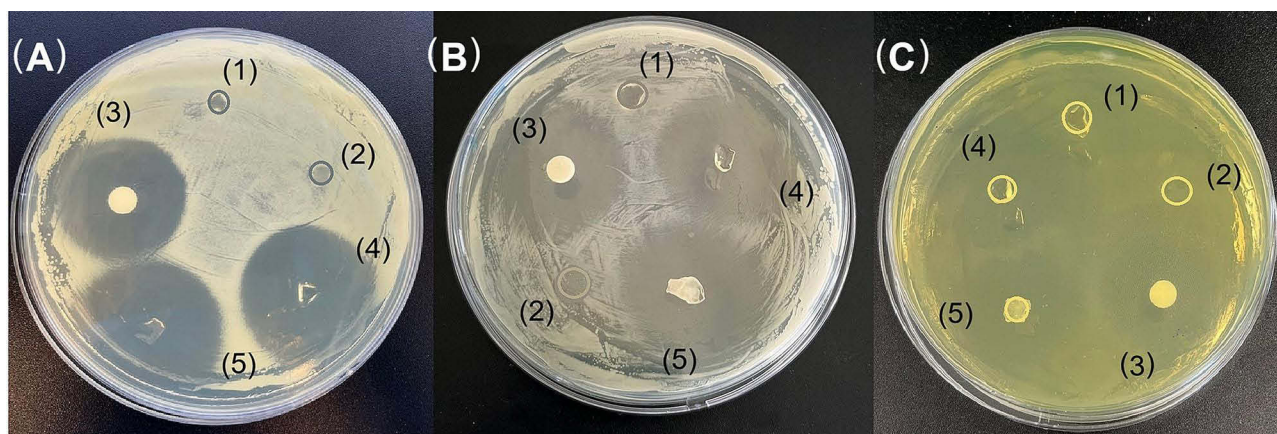


Figure 6 Disc diffusion testing toward (A) *S. aureus*, (B) *K. pneumoniae* and (C) *P. aeruginosa*. Samples (1) Alg; (2) Alg-ZnBSA; (3) CF; (4) Alg-CF and (5) Alg-ZnBSA-CF.

98%, 84%, 58% and 53% as the concentration increased. The calculated IC_{50} after 72 hours was 300.6 $\mu\text{g/mL}$, indicating a relatively high toxicity threshold (Figure 5C). Notably, this cytotoxic effect was not observed at 24 or 48 hours, suggesting a time-dependent mechanism, possibly due to intracellular accumulation or gradual pH alteration in the medium. To enable a quantitative comparison across all time points, numerical estimation of IC_{50} values for 24 and 48 hours was performed by extrapolating the experimentally obtained dose-response curves to zero cell viability. Based on this theoretical extrapolation, the estimated IC_{50} values were approximately 737.1 $\mu\text{g/mL}$ at 24 hours and 573.4 $\mu\text{g/mL}$ at 48 hours. Interestingly, when comparing the effects of CF-HCl on RAW 264.7 cells and BJ fibroblasts, RAW macrophages showed greater sensitivity to CF-induced stress. This is likely attributable to their immunological role and heightened reactivity to environmental changes, such as acidification. Since CF-HCl lowers the pH of the culture medium, immune cells such as RAW 264.7, which are more metabolically active and responsive to inflammatory cues, may be more vulnerable to such stress than nonphagocytic fibroblasts.

Remarkably, the release profile of ZnBSA-CF in cell medium with the addition of FBS (see Figure S13) showed a completely different trend compared to PBS pH 7.4 (see Figure S10) and E3 (see Figure S8). The initial burst of 16% (0.03 mg of CF/mL) at $T=0$ was then reduced to approximately 7% (0.015 mg of CF/mL) after 24 h, which remained stable throughout the experiment. This effect could be attributed to the adsorption of serum proteins and the formation of a stabilizing protein corona, which not only blocked further CF release but also promoted partial drug re-adsorption on the nanoflower surface. Consistent with this, DLS measurements confirmed the enhanced dispersion stability of ZnBSA-CF in the FBS-containing medium, with a reduced hydrodynamic size ($\sim 592 \pm 61$ nm) and a moderately negative surface charge (-11.7 mV). This protein-driven modulation transforms ZnBSA-CF into a slow-release system in a physiological environment, explaining the excellent cytocompatibility observed in fibroblasts and macrophages (>90% viability, Figure 5; Figures S10 and S11). These findings emphasize the critical role of cell type in in vitro toxicity studies, as

Table 1 The Results of Disc Diffusion Testing

Sample \ Species	<i>S. aureus</i>	<i>K. pneumoniae</i>	<i>P. aeruginosa</i>
	Radius of Inhibition Zone (mm)		
(1) Alg	0	0	0
(2) Alg-ZnBSA	0	0	0
(3) CF	25	29	29
(4) Alg-CF	27	26	26
(5) Alg-ZnBSA-CF	31	30	30

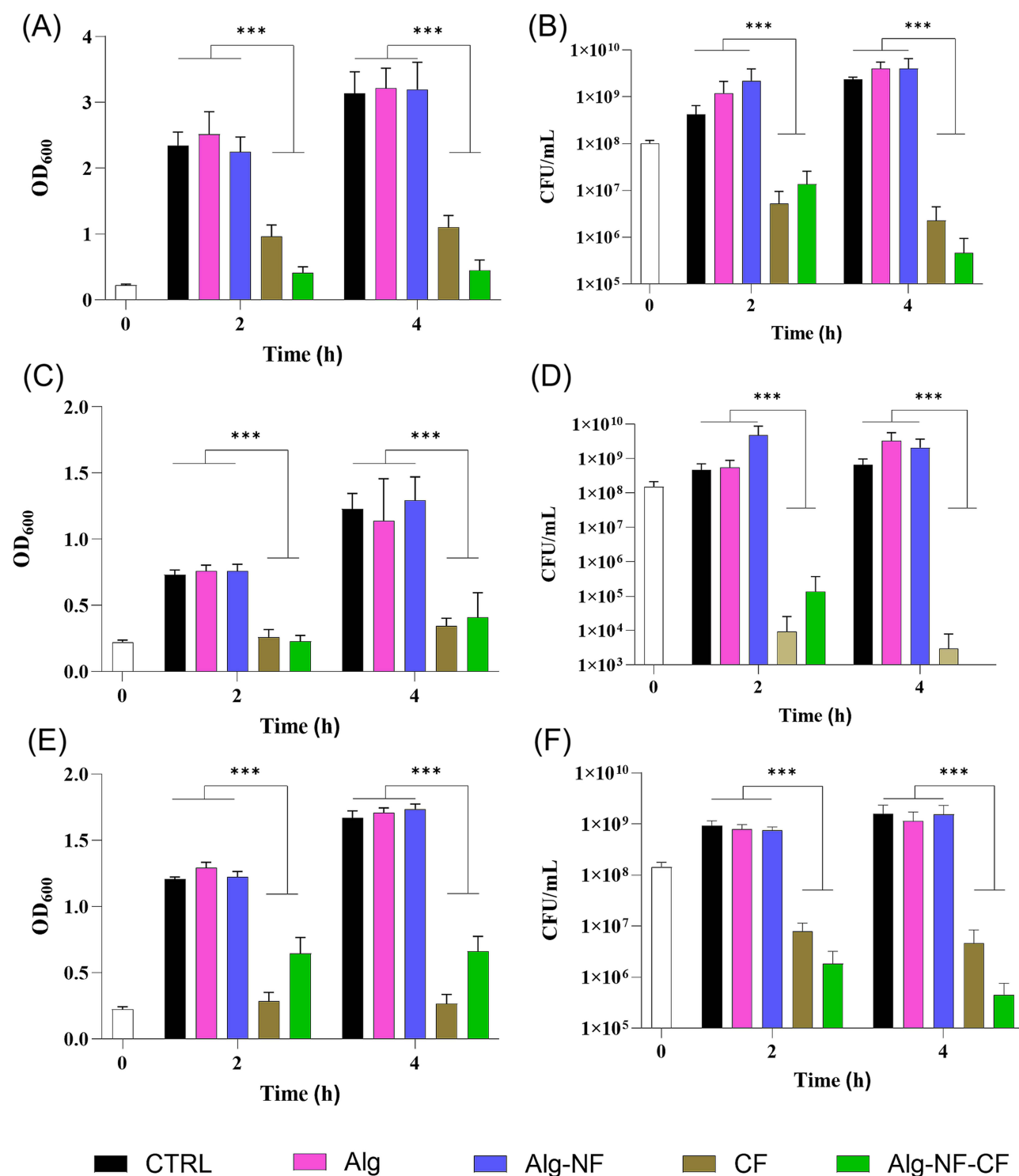


Figure 7 Antibacterial activity of hydrogel-based nanoflowers. Growth of *S. aureus* (A and B), *P. aeruginosa* (C and D) and *K. pneumoniae* (E and F) assessed by OD₆₀₀ (A, C, E) and CFU/mL (B, D, F). Bars show baseline (0 h, white) and cultures at 2 h and 4 h under five conditions: untreated (black), Alg (pink), Alg-ZnBSA (blue), CF (brown) and ZnBSA-CF (green). Alg and Alg-ZnBSA had no effect, whereas ZnBSA-CF markedly inhibited bacterial growth, comparable to or exceeding free CF. Data are presented as the mean ± SD (n = 3 independent replicates per group). Statistical analysis was performed separately for each time point using one-way analysis of variance (ANOVA) followed by Tukey's multiple-comparisons test. Statistical significance is indicated as ***p < 0.001; not significant changes were not included.

different physiological functions can lead to markedly distinct responses. Moreover, the ZnBSA-CF nanoformulation clearly mitigated the toxicity of CF, presumably by slowing its release and buffering the local environment, thus reducing abrupt pH shifts and minimizing cytotoxic stress in sensitive cells. This underscores the therapeutic benefit of nanoencapsulation strategies not only in controlled drug delivery but also in enhancing cellular tolerance and safety, especially for long-term biomedical applications.

Antimicrobial Testing of ZnBSA-CF Nanostructures

Ciprofloxacin, an antibiotic used in osteomyelitis treatment, was embedded in ZnBSA nanostructures as described in the Methods section to obtain the ZnBSA-CF sample. Pure alginate gel (Alg) and alginate gel incorporating ZnBSA (Alg-ZnBSA) or CF (Alg-CF) were also prepared as described in the Methods. All material samples were subjected to antibacterial evaluation using three complementary assays: (i) disc diffusion, (ii) optical density measurements in liquid cultures, and (iii) quantification of viable bacterial cells in liquid media based on colony-forming units (CFU). The results of disc diffusion testing toward *S. aureus*, *K. pneumoniae* and *P. aeruginosa* are shown in Figure 6A–C and summarized in Table 1. Alg and Alg-ZnBSA samples served as carrier-component controls to assess their influence on bacterial growth (negative controls), whereas Alg-CF and CF were used as positive controls to confirm the antibiotic effect (positive control). As expected, Alg and Alg-ZnBSA exhibited no antibacterial activity, with no inhibition zone observed. It can be clearly seen that CF shows antimicrobial activity against the studied species (positive control – Alg-CF and CF), and its activity is not hindered by the drug delivery system (Alg-ZnBSA-CF). For the Alg-ZnBSA-CF sample, the inhibition zone was the highest among the studied bacterial species, which proves efficient CF release from the proposed drug carrier system.

Next, we evaluated the antibacterial activity of the obtained materials against bacterial cultures. Two parameters were monitored during the growth of *S. aureus*, *P. aeruginosa* and *K. pneumoniae*: optical density at 600 nm (OD₆₀₀; Figure 7A, C, E) and viable cell counts (CFU/mL; Figure 7B, D, F). Consistent with the disc diffusion assay, neither the hydrogel matrix (Alg) nor the unloaded nanoflowers (Alg-ZnBSA) affected bacterial growth. In contrast, ZnBSA nanoflowers loaded with CF (ZnBSA-CF) exerted a pronounced bactericidal effect, comparable to or exceeding that of free CF.

Conclusion

In summary, this study reports the successful development of zinc–bovine serum albumin (ZnBSA) hybrid nanostructures as a promising localized drug delivery system for osteomyelitis treatment. A reproducible protocol for ZnBSA nanoflower synthesis was established, and the effects of BSA and PBS concentration, volume, and reaction time on the resulting morphology and composition were systematically investigated. Hybrid nanostructures composed of BSA and Zn₃(PO₄)₂·H₂O were obtained after 4 hours of reaction in 10 mM PBS with varying BSA concentrations. The resulting ZnBSA carriers exhibit rapid synthesis, excellent biocompatibility, and efficient antibiotic loading and release properties. To the best of our knowledge, this is the first study to evaluate the toxicity of ZnBSA nanoflowers both in vitro and in vivo, confirming their overall safety as ciprofloxacin carriers. These findings advance the development of safe and effective nanostructured platforms for localized antibacterial therapy in osteomyelitis and lay the groundwork for future studies, including mammalian models.

Ethics Approval and Consent to Participate

Research involving fish embryos up to 5 days (120 h) postfertilization and commercially available in vitro cell cultures does not require ethics committee approval. The mouse blood used in this study was obtained for research purposes only, with no procedures performed on live animals, and therefore did not require additional ethics committee approval. All in accordance with Directive 2010/63/EU of the European Parliament and of the Council, as well as the Polish Act of 15 January 2015 on the protection of animals used for scientific or educational purposes (Dz.U. 2015 poz. 266, with subsequent amendments). All bacterial isolation swabs were obtained by veterinarians during routine clinic visits. Each time, written consent from the pet's owner to take a swab and use the isolated bacteria in scientific activities was

received. According to Directive 2010/63/EU of the European Parliament and Council, ethical approval is not required for the use of clinical isolates isolated from dogs.

Acknowledgment

Kamila Sadowska would like to acknowledge financial support to Polish Ministry of Science and Higher Education, Program Science for the Society project number NdS-II/SP/0335/2023/01.

Author Contributions

All authors made a significant contribution to the work reported, whether in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

References

- Schmitt SK. Osteomyelitis. *Infect Dis Clin North Ame*. 2017;31(2):325–338. doi:10.1016/j.idc.2017.01.010
- Kavanagh N, Ryan EJ, and Widaa A, et al. Staphylococcal osteomyelitis: disease progression, treatment challenges, and future directions. *Clin Microbiol Rev*. 2018;31(2):e00084–17. doi: 10.1128/CMR.00084-17
- Urish KL, Cassat JE, Ottemann KM. *Staphylococcus aureus* osteomyelitis: bone, bugs, and surgery. *Infect Immun*. 2020;88(7). doi:10.1128/IAI.00932-19
- Seghrouchni K, Van Delden C, Dominguez D, et al. Remission after treatment of osteoarticular infections due to *Pseudomonas aeruginosa* versus *Staphylococcus aureus*: a case-controlled study. *Int Orthop*. 2012;36(5):1065–1071. doi:10.1007/s00264-011-1366-8
- Tice AD. Risk factors and treatment outcomes in osteomyelitis. *J Antimicrob Chemother*. 2003;51(5):1261–1268. doi:10.1093/jac/dkg186
- Murray CJL, Ikuta KS, Sharara F, et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *Lancet*. 2022;399(10325):629–655. doi:10.1016/S0140-6736(21)02724-0
- Chen Y-C, Gad SF, Chobisa D, Li Y, Yeo Y. Local drug delivery systems for inflammatory diseases: status quo, challenges, and opportunities. *J Control Release*. 2021;330:438–460. doi:10.1016/j.jconrel.2020.12.025
- Nandi SK, Bandyopadhyay S, Das P, et al. Understanding osteomyelitis and its treatment through local drug delivery system. *Biotechnol Adv*. 2016;34(8):1305–1317. doi:10.1016/j.biotechadv.2016.09.005
- Bobrowska K, Sadowska K, Stolarczyk K, Przeźniak-Welenc M, Golec P, Bilewicz R. Bovine serum albumin – hydroxyapatite nanoflowers as potential local drug delivery system of ciprofloxacin. *Int J Nanomed*. 2023;18:6449–6467. doi:10.2147/IJN.S427258
- Ge J, Lei J, Zare RN. Protein–inorganic hybrid nanoflowers. *Nat Nanotechnol*. 2012;7(7):428–432. doi:10.1038/nnano.2012.80
- Jafari-Nodoushan H, Mojtavani S, Faramarzi MA, Samadi N. Organic–inorganic hybrid nanoflowers: the known, the unknown, and the future. *Adv Colloid Interface Sci Elsevier B V*. 2022;309. doi:10.1016/j.cis.2022.102780
- Bilal M, Asgher M, Shah SZH, Iqbal HMN. Engineering enzyme-coupled hybrid nanoflowers: the quest for optimum performance to meet biocatalytic challenges and opportunities. *Int J Biolog Macromole*. 2019;135:677–690. doi:10.1016/j.ijbiomac.2019.05.206
- Zhang B, Li P, Zhang H, et al. Preparation of lipase/Zn3(PO4)2 hybrid nanoflower and its catalytic performance as an immobilized enzyme. *Chem Eng J*. 2016;291:287–297. doi:10.1016/j.cej.2016.01.104
- Jamal HS, Raja R, Ahmed S, Yesiloz G, Ali SA. Immobilization of collagenase in inorganic hybrid nanoflowers with enhanced stability, proteolytic activity, and their anti-amyloid potential. *Int J Biol Macromol*. 2024;274. doi:10.1016/j.ijbiomac.2024.133114
- Kiani M, Mojtavani S, Jafari-Nodoushan H, Tabib S-R, Hassannejad N, Faramarzi MA. Fast anisotropic growth of the biomineralized zinc phosphate nanocrystals for a facile and instant construction of laccase@Zn3(PO4)2 hybrid nanoflowers. *Int J Biol Macromol*. 2022;204:520–531. doi:10.1016/j.ijbiomac.2022.02.023
- Luo M-L, Chen H, Chen G-Y, Wang S, Wang Y, Yang F-Q. Preparation of alcohol dehydrogenase–zinc phosphate hybrid nanoflowers through biomimetic mineralization and its application in the inhibitor screening. *Molecules*. 2023;28(14):5429. doi:10.3390/molecules28145429
- Wang Z, Liu P, Fang Z, Jiang H. Trypsin/Zn3(PO4)2 hybrid nanoflowers: controlled synthesis and excellent performance as an immobilized enzyme. *Int J Mol Sci*. 2022;23(19). doi:10.3390/ijms231911853
- Iranmanesh S, Badoei-Dalfard A, Karami Z. Synthesis and characterization of glucose oxidase bimetallic hybrid magnetic nanoflowers for a sensitive glucose biosensor and an effective antibacterial agent. *Biochem Eng J*. 2025;214. doi:10.1016/j.bej.2024.109583
- Zhang B, Li P, Zhang H, et al. Red-blood-cell-like BSA/Zn3(PO4)2 hybrid particles: preparation and application to adsorption of heavy metal ions. *Appl Surf Sci*. 2016;366:328–338. doi:10.1016/j.apsusc.2016.01.074
- Section 2 effects on biotic systems. 2025. Available from: <http://www.oecd.org/termsandconditions/>. Accessed January 27, 2026.
- Herschke L, Enkelmann V, Lieberwirth I, Wegner G. The role of hydrogen bonding in the crystal structures of zinc phosphate hydrates. *Chem Euro J*. 2004;10(11):2795–2803. doi:10.1002/chem.200305693
- Spiegelhoff Y, Zemajtis F, Kheirandish E, et al. Enhanced UV light-emission of zinc-phosphate-hydrate hydrothermally-grown on Cu metal substrates for opto-electronic applications. *Adv Electron Mater*. 2023;9(10). doi:10.1002/aelm.202300272

23. Bach S, Celinski VR, Dietzsch M, et al. Thermally highly stable amorphous zinc phosphate intermediates during the formation of zinc phosphate hydrate. *J Am Chem Soc.* **2015**;137(6):2285–2294. doi:10.1021/ja5103663
24. Jones A, Zeller MA, and Sharma S. Thermal, Mechanical, and Moisture Absorption Properties of Egg White Protein Bioplastics with Natural Rubber and Glycerol. *Prog Biomater.* **2013**;2(1):12. doi:10.1186/2194-0517-2-12
25. Rivera A, Valdés L, Jiménez J, et al. Smectite as ciprofloxacin delivery system: intercalation and temperature-controlled release properties. *Appl Clay Sci.* **2016**;124-125:150–156. doi:10.1016/j.clay.2016.02.006
26. Dumez H, Reinhart WH, Guetens G, De Bruijn EA. Human red blood cells: rheological aspects, uptake, and release of cytotoxic drugs. *Crit Rev Clin Laborator Sci.* **2004**;41(2):159–188. doi:10.1080/10408360490452031
27. Dymek K, Kurowski G, Kuterasiński Ł, et al. In search of effective UiO-66 metal–organic frameworks for artificial kidney application. *ACS Appl Mater Interfaces.* **2021**;13(38):45149–45160. doi:10.1021/acsami.1c05972
28. Maslyk M, Janeczko M, Demchuk OM, et al. A representative of arylcyanomethylenequinone oximes effectively inhibits growth and formation of hyphae in *Candida albicans* and influences the activity of protein kinases *in vitro*. *Saudi Pharm J.* **2018**;26(2):244–252. doi:10.1016/j.jsps.2017.12.004

Nanotechnology, Science and Applications

Dovepress
Taylor & Francis Group

Publish your work in this journal

Nanotechnology, Science and Applications is an international, peer-reviewed, open access journal that focuses on the science of nanotechnology in a wide range of industrial and academic applications. It is characterized by the rapid reporting across all sectors, including engineering, optics, bio-medicine, cosmetics, textiles, resource sustainability and science. Applied research into nano-materials, particles, nano-structures and fabrication, diagnostics and analytics, drug delivery and toxicology constitute the primary direction of the journal. The manuscript management system is completely online and includes a very quick and fair peer-review system, which is all easy to use. Visit <http://www.dovepress.com/testimonials.php> to read real quotes from published authors.

Submit your manuscript here: <https://www.dovepress.com/nanotechnology-science-and-applications-journal>