



Nisin Z and cinnamaldehyde co-delivery via fiber-reinforced alginate hydrogels: A synergistic *in vitro* strategy against infected pressure ulcers

Joana Fernandes^{a,1}, Micaela Gomes^{a,1}, Ana Rita C. Bastos^a, Sonia L.C. Pinho^{b,c,d}, Helena P. Felgueiras^{a,*}

^a Centre for Textile Science and Technology (2C2T), Department of Textile Engineering, University of Minho, Campus of Azurém, 4800-058, Guimarães, Portugal

^b Center for Neuroscience and Cell Biology (CNC), Centre for Innovative Biomedicine and Biotechnology (CIBB), University of Coimbra, 3004-517, Coimbra, Portugal

^c Research Centre for Natural Resources, Environment and Society (CERNAS), Coimbra Agriculture School, Polytechnic of Coimbra, 3045-601, Coimbra, Portugal

^d Vasco da Gama Research Center (CIVG), Vasco da Gama University School, 3020-210, Coimbra, Portugal

ARTICLE INFO

Keywords:

Fiber-reinforced hydrogels
Electrospun films
Hydrolyzed fiber fragments
Dual antimicrobial action
Chronic wound management

ABSTRACT

Infected pressure ulcers remain difficult to manage because conventional dressings cannot simultaneously provide mechanical robustness, sustained antimicrobial delivery, and a favorable healing environment. Here, sodium alginate (SA) hydrogels were engineered with a dual-reinforcement strategy, electrospun polycaprolactone (PCL) fibrous films versus their hydrolysis-derived fragments, to independently tune mechanical behavior while compartmentalizing two bioactive agents, Nisin Z (NZ) and cinnamaldehyde (CN), for combined antimicrobial and antioxidant function. This architecture-driven approach distinguishes the system from previous single-reinforcement or single-agent SA-based dressings.

NZ was highly active against Gram-positive bacteria (*S. aureus*, *S. epidermidis*; MBC = 8 µg/mL) and less so against Gram-negative strains (*E. coli*, 128 µg/mL; *P. aeruginosa*, 32 µg/mL), with CN requiring 20–320 µg/mL. Combined, NZ and CN acted synergistically against *S. aureus* (FICI = 0.019). Film-reinforced hydrogels preserved compressive strength, while fragment-reinforced systems favored flexibility and recovery after 50% deformation, giving the platform tunable mechanics for different wound geometries. NZ and CN were released over 48 h through diffusion- and retention-dominated mechanisms, respectively, sustaining >90% bacterial reduction against Gram-positive strains, strong antibiofilm activity, and > 80% DPPH antioxidant scavenging, without compromising HaCaT keratinocyte viability (> 85%).

By showing that reinforcement architecture and bioactive compartmentalization can be engineered jointly rather than independently, this work introduces a versatile SA-based platform for infected chronic wound management, with direct translational potential for pressure ulcer care.

1. Introduction

Pressure ulcers, or pressure injuries, are localized lesions of the skin and underlying tissues, caused by sustained pressure, shear forces, or friction, particularly in immobilized and elderly patients. These wounds represent a significant clinical and economic burden due to their high prevalence and association with severe complications [1–3]. Their management remains challenging, as chronic wounds provide a favorable environment for microbial colonization and biofilm formation, which significantly impair tissue regeneration and reduce the efficacy of conventional antimicrobial therapies [4–6]. Also, common dressings,

such as gauzes and simple polymeric films, often fail to maintain an optimal moist environment, deliver bioactive agents effectively, and adapt to irregular wound surfaces, thereby hindering their therapeutic potential [7,8].

In this context, hydrogels have emerged as promising wound care materials due to their high-water content, biocompatibility, and tunable physicochemical properties [9]. By mimicking the extracellular matrix, they provide a moist environment that supports cell migration and proliferation, while facilitating gas exchange and nutrient transport [10,11]. Moreover, hydrogels enable the localized and controlled release of antimicrobial and regenerative agents, enhancing therapeutic

* Corresponding author.

E-mail address: helena.felgueiras@2c2t.uminho.pt (H.P. Felgueiras).

¹ Authors contributed equally to this work.

efficacy and minimizing systemic side effects [12]. Among naturally derived polymers, sodium alginate (SA) has been widely used in wound management due to its biocompatibility, high exudate absorption capacity, and ability to form stable hydrogels under mild conditions, enabling effective moisture retention and controlled drug delivery [13]. However, conventional hydrogels often exhibit limited mechanical strength and structural stability, restricting their use in load bearing or highly exudative wounds [14].

To address these limitations, reinforcement strategies have increasingly focused on incorporating electrospun fibers. Electrospinning enables the production of ultrafine fibers with high surface area, interconnected porosity, and controllable architecture, also closely resembling the native extracellular matrix fibrous system [15,16]. These fibrous structures support cell adhesion and migration while acting as physical barriers against microbial invasion [17–19]. Polycaprolactone (PCL) is particularly suitable for reinforcing hydrogel matrices due to its biocompatibility, tunable degradation rate, and mechanical resilience [20,21]. Fragmented PCL fibers embedded within hydrogel matrices can form composite systems that combine hydration and flexibility with enhanced mechanical stability, resulting in improved tensile properties and resistance to deformation [22–25]. In addition, fibrous networks can act as reservoirs for therapeutic agents, enabling sustained and localized therapeutic delivery [17,19].

The incorporation of bioactive agents in fiber-reinforced hydrogels represents a promising strategy to fight infections and promote tissue regeneration in chronic wounds. Composite systems loaded with antibiotics, essential oils, natural extracts, or growth factors have demonstrated sustained antibacterial activity and accelerated wound closure [11,26–28]. In this context, Nisin Z (NZ), a 34-amino acid amphipathic peptide, exhibits potent activity against Gram-positive bacteria through inhibition of cell wall synthesis and membrane pore formation, combined with high stability and low cytotoxicity [29–32]. Conversely, cinnamaldehyde (CN), a major component of *Cinnamomum* essential oils, displays broad-spectrum antimicrobial, antifungal, and anti-inflammatory activities, primarily through membrane disruption and enzyme inhibition. Its stabilization within polymeric matrices enables controlled release and preservation of bioactivity [26,33–35].

Despite the growing interest in dual-agent hydrogels for infected wound management, most existing formulations present important design limitations. In many cases, both bioactive agents are loaded into the same hydrogel matrix, which restricts independent control over their release kinetics and often results in premature burst release of the more soluble compound while compromising the stability of the more labile one [36]. Pairing hydrophilic antimicrobial peptides with hydrophobic essential oil components in a single carrier further complicates this balance, as the two agents differ markedly in solubility, stability, and diffusion behavior. When fiber reinforcement is included, it is typically limited to a mechanical role, with little exploration of its potential as a distinct compartment for housing one of the bioactive agents independently [37].

This lack of independent, compartmentalized control over the loading and release of chemically distinct antimicrobial agents represents a clear gap in the current design of dual-agent hydrogels. To date, no studies have reported a system that combines electrospun fiber reinforcement, used as an independent bioactive compartment, with the dual delivery of NZ and CN within alginate hydrogels. Hence, this work proposes a new design strategy for multifunctional wound dressings by integrating electrospun PCL reinforcement with the compartmentalized delivery of NZ and CN within alginate hydrogels. This previously unreported approach combines structural reinforcement with complementary antibacterial and antioxidant functions, enabling simultaneous optimization of mechanical performance and biological activity for the treatment of infected pressure ulcers.

2. Materials and methods

2.1. Materials

Polycaprolactone (PCL) (Mw 80,000) was purchased from Sigma-Aldrich, Merck KGaA (Germany), whereas reagents *N,N*-dimethylformamide (DMF; $\rho = 0.948$ kg/L) and chloroform (CHF; solubility of 8 g/L in distilled water or dH₂O) were obtained from PanReac AppliChem, ITW Reagents (Italy). These were used in the preparation of polymer solutions to produce fibrous films. Sodium alginate (SA), cinnamaldehyde (CN), and Nisin Z (NZ) were purchased from Sigma-Aldrich Merck KGaA (Germany) and were used to produce the hydrogels and provide bioactivity to the system, respectively. Calcium chloride (CaCl₂) purchased from ChemLabs (South Africa) was used as a coagulation bath for the formation of hydrogels.

Mueller Hinton broth (MHB, 21 g/L), purchased from Pronadisa (Condalab, Spain), trypticase soy broth (TSB, 30 g/L), nutrient broth (NB, 13 g/L), and agar (for trypticase soy agar, TSA, and nutrient agar, NA, preparation) purchased from HiMedia Laboratories (Germany) were used as culture media for bacterial growth. *Staphylococcus aureus* (*S. aureus*, ATCC 6538), *Staphylococcus epidermidis* (*S. epidermidis*, ATCC 35984), *Pseudomonas aeruginosa* (*P. aeruginosa*, ATCC 27853), and *Escherichia coli* (*E. coli*, ATCC 25922), the reference bacterial strains, were obtained from the American Type Culture Collection (ATCC, USA).

Phosphate-buffered saline solution (PBS) was prepared at pH 7.4 (adjusted with 1 M hydrochloric acid) from a combination of sodium chloride (8.00 g/L), potassium chloride (0.20 g/L), dibasic sodium phosphate (1.44 g/L), and monopotassium phosphate (0.24 g/L), all obtained from Sigma-Aldrich.

Folin-Ciocalteu reagent, gallic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid; 97.0%) were purchased from Sigma-Aldrich (Germany). Sodium carbonate was purchased from Riedel-de Haën, (Germany), and sodium hydroxide from Labkem (Spain). Dimethyl sulfoxide (DMSO) and ethanol (99.8%) were purchased from Fisher Scientific (UK). Acetic acid was purchased from Panreac (Spain). Crystal violet was purchased from Certistain (USA). The human keratinocyte cell line (HaCaT) was purchased from Cell Lines Service GmbH (CLS, Germany). Dulbecco's Modified Eagle Medium (DMEM, high glucose, GlutaMAX, pyruvate), antibiotic-antimycotic solution (penicillin-streptomycin; 10,000 U/mL), and trypsin-EDTA solution (0.25% w/v) were all obtained from VWR Chemicals (Portugal). Methanol, tris(hydroxymethyl)amino-methane (TRIS), fetal bovine serum (FBS), trypan blue, sodium resazurin, sulforhodamine B (SRB), and Triton X-100 were supplied by Merck (Portugal), while Hoechst 33342 was purchased from Thermo Fisher Scientific (Portugal).

The reagents and polymers were all used as commercially available, without additional chemical modifications.

2.2. Methods

2.2.1. Antimicrobial evaluation and synergy of active agents

The antimicrobial activity of NZ and CN was evaluated individually against *S. aureus*, *S. epidermidis*, *E. coli*, and *P. aeruginosa* by determining the minimum inhibitory (MIC) and minimum bactericidal (MBC) concentrations, using broth microdilution followed by colony counting on agar (Section S1-S1.1 of Supporting Information file). The interaction between NZ and CN was assessed using the checkerboard method, and the fractional inhibitory concentration index (FICI) was calculated (Eq. (1)) to classify the interaction as synergistic (FICI ≤ 0.5), additive (0.5–1.0), indifferent (1.0–4.0), or antagonistic (> 4.0). Full checkerboard protocol details are provided in Section S1-S1.2 of Supporting Information file.

$$FICI = \frac{MBC_{NZ \text{ combined}}}{MBC_{NZ \text{ isolated}}} + \frac{MBC_{CN \text{ combined}}}{MBC_{CN \text{ isolated}}} \quad (1)$$

2.2.2. Total phenolic content and antioxidant activity of the free agents

Total phenolic compound content (TPC) of NZ and CN was determined by the Folin-Ciocalteu method [38], expressed as mg gallic acid equivalents per gram of sample. Antioxidant activity was evaluated by the DPPH radical scavenging assay [39], expressed as percentage of DPPH reduced (%DPPHr) relative to Trolox as positive control. Full protocols, reagent concentrations, and calculation equations are provided in Supporting Information, respectively in Sections S1-S1.3 and S1-S1.4.

2.2.3. Production of PCL films by electrospinning

PCL polymer solution was prepared at 14% (w/v) in CHF/DMF at 70/30% (v/v) at a stirring speed of 150 rpm at 50 °C for 4 h. Viscosity measurements were performed on a Brookfield DV-II + Pro viscometer (Hadamar-Steinbach, Germany), using shaft 21, at 15 rpm, for approximately 10 min at 22 °C, with recordings every 30 s, while conductivity was measured using Thermo Scientific Benchtop Meter equipment (Orion Versa Star Pro, Waltham, MA, USA).

Electrospun films were continuously deposited on an aluminum foil collector for 1.5 h. The processing parameters were optimized for a voltage of 25 kV, flow rate of 0.8 mL/min, and distance from the needle to the collector of 18 cm, using a needle with an internal diameter of 22 gauge. Throughout the process, environmental conditions were controlled, maintaining constant temperature (20–25 °C) and humidity (40–60%) constant [40].

2.2.4. Hydrolysis and fragmentation of PCL fibers

Electrospun films were detached from the aluminum collector and cut into fragments smaller than $1 \times 1 \text{ cm}^2$. They were then washed with 99% ethanol, dried, and hydrolyzed into fragments by treatment with 1 M NaOH for 3 days. After the treatment period, the pH of the suspension was evaluated with an indicator strip. The suspension was then centrifuged at 4000 rpm for 10 min to separate the fragmented fibers from the liquid. To ensure complete removal of NaOH residues, the fragments were washed successively in 99% ethanol, with the pH measured after each wash until it stabilized. Finally, the fragments were dried at 37 °C and stored in a desiccator until further use.

2.2.5. Modification of electrospun films and fragments with CN

PCL fragments and films were submerged in CN solutions prepared in ethanol at different concentrations: MBC, $5 \times \text{MBC}$, $10 \times \text{MBC}$, $20 \times \text{MBC}$, $50 \times \text{MBC}$, and $100 \times \text{MBC}$, to ensure that the amount effectively bound to the fragments and films was equal to or greater than the determined MBC. The samples were kept under agitation for 24 h. The concentration of CN immobilized in the fibers was determined indirectly by differences between UV–visible spectroscopy absorbance readings of the amount of CN at 0 h of contact (stock solution) and the amount remaining after immersion of the fibers. Experiments were done in triplicate.

2.2.6. Preparation of SA hydrogels

Hydrogels were produced from a 2% (m/v) SA solution in dH₂O, heated to 50 °C at 150 rpm for 12–18 h. The SA solution was then transferred to glass Petri dishes (90 mm in diameter) at 20 mL (ideal volume to obtain a thin, continuous hydrogel that covered the entire bottom of the dish) and left at room temperature for $\approx 2 \text{ h}$ to stabilize (homogeneous distribution of the solution without bubbles). Next, 2% (m/v) CaCl₂ was added, also at 20 mL, to induce coagulation. The bath was left to act for 2 h30, after which the hydrogels were detached and washed successively in dH₂O to eliminate any remaining salts [24].

2.2.7. Loading of hydrogels with NZ

After optimizing the production of the hydrogels, the functionalization process began. For this, NZ was dissolved in dH₂O at different concentrations: MBC, $10 \times \text{MBC}$, $20 \times \text{MBC}$, $30 \times \text{MBC}$, $50 \times \text{MBC}$, and

$100 \times \text{MBC}$ to ensure that the amount effectively bound to the hydrogels was equal or greater than MBC. Hydrogel samples were immersed and kept under agitation for 24 h. After this period, absorbance readings were taken by UV–visible spectroscopy. The concentration of NZ bound to the hydrogel was determined indirectly, by the difference between the amount of NZ at 0 h of contact (stock solution) and the amount remaining after immersion of the hydrogel. Experiments were conducted in triplicate.

To evaluate the antibacterial efficacy of the hydrogels and determine the optimal concentration of NZ, a “shake flask” test was performed based on an adaptation of the ASTM-E2149–01 standard. This test was conducted against suspensions of *S. aureus*, *S. epidermidis*, *E. coli*, and *P. aeruginosa* prepared in TSB and NB at $1 \times 10^5 \text{ CFU/mL}$. Quadrangular samples of the NZ-loaded and unloaded (control) hydrogels with 6 cm sides were placed in contact with 500 μL of the bacterial suspension and incubated for 24 h at 37 °C at 120 rpm. After this time, 20 μL aliquots of the suspension were collected, and successive dilutions were performed in PBS (between 10^{-1} and 10^{-5}). The dilutions were then cultured in Petri dishes (90 mm in diameter) containing TSA or NA and incubated at 37 °C for 18 to 24 h. After incubation, the colonies formed were counted, and the results were expressed as percentage of bacterial growth inhibition. The results obtained allowed the selection of the optimal concentration to be used for the modification of the hydrogels.

It should be noted that this preliminary antibacterial test was not applied to the fragments and fibrous films after modification with CN, described in Section 2.2.6, due to the high surface area of these structures, which tends to increase the exposure of the agent. Additionally, the fibrous structures were not designed for direct exposure to bacteria, but rather the hydrogel, where these fibers were incorporated. For that reason, this step was deemed non-essential for deciding on the functionalization concentration of the agent.

2.2.8. Fiber-reinforced hydrogels

After optimizing the production conditions and defining the ideal concentrations for functionalizing the bioactive agents in each structural component, non-reinforced (controls) and reinforced hydrogels with and without active agents were produced. The agents were incorporated by immersing the different structures in the respective active solutions, under constant agitation as described in Sections 2.2.6 and 2.2.8. Finally, the hydrogels with and without fibrous reinforcement were washed in dH₂O to remove coagulation bath salts that remained attached and then disinfected in sequential washes with 70% ethanol ($1 \times$) and PBS ($3 \times$), each washing lasting 5 min under constant agitation at 120 rpm.

The incorporation of the reinforcing structures followed distinct procedures depending on their morphology. For film-reinforced hydrogels (SA/FL), the PCL film was cut to match the dimensions of the Petri dish used for casting and placed at the bottom of the dish prior to pouring the SA solution, which was then cast directly over it following the procedure described in Section 2.2.6. For fragment-reinforced hydrogels (SA/FR), the mass of PCL film used per casting dish was first determined and used as a reference to define an equivalent mass of fragments for each formulation, ensuring comparable PCL content between the two reinforcement types. The fragments were then dispersed directly into the SA solution under magnetic stirring for 30 min, followed by ultrasonication for an additional 10 min to improve homogeneity, prior to casting. In both cases, the resulting solutions were left to stabilize at room temperature and subsequently coagulated with the CaCl₂ bath, following the conditions described in Section 2.2.6.

For the nomenclature of the samples, listed in Table 1, the symbol “/” was used to indicate combination of structures in the system and the symbol “+” to indicate the addition of the active agents. For simplicity, electrospun PCL films were designated FL, while the fragments resulting from the hydrolysis of these films were identified as FR. The hydrogel, being a structure that is repeated in all samples, was designated by the name of the polymer, SA.

Table 1
Sample code.

Code	Reinforcement	NZ	CN
SA	–	–	–
SA/FL	Film	–	–
SA/FR	Fragments	–	–
SA + NZ	–	✓	–
SA + NZ/FL	Film	✓	–
SA + NZ/FR	Fragments	✓	–
SA/FL + CN	Film	–	✓
SA/FR + CN	Fragments	–	✓
SA + NZ/FL + CN	Film	✓	✓
SA + NZ/FR + CN	Fragments	✓	✓

2.2.9. Physical, chemical, thermal, and mechanical characterization

Individual system components (polymers, bioactive agents, films, fiber fragments, and hydrogels) were characterized as controls alongside the corresponding composite systems. Morphology was examined by scanning electron microscopy (SEM). Chemical composition was analyzed by Fourier-transform infrared spectroscopy with attenuated total reflectance (ATR-FTIR) (400–4000 cm^{-1}), while thermal behavior was evaluated by thermogravimetric analysis (TGA/DTG) and differential scanning calorimetry (DSC). Physicochemical properties, including hydration degree, degradation profile, porosity, and air permeability, were determined using gravimetric or standardized methods (ISO 9237 for air permeability). Mechanical performance was assessed by adhesiveness and cyclic compression tests (three cycles at 50% deformation) using a Hitachi DMA 7100. Detailed experimental procedures, equipment settings, sample dimensions, and calculation methods are provided in the Supporting Information (Section S1-S1.5).

2.2.10. Biological evaluations

The biological performance of the developed hydrogels was evaluated through bioactive release, antioxidant, antibacterial, antibiofilm, and cytocompatibility assays. Detailed experimental procedures, reagent concentrations, instrument settings and calculation equations are provided in the Supporting Information file, Section S1-S1.6.

The release kinetics of NZ and CN from control and reinforced hydrogels were determined under sink conditions by ultraviolet-visible (UV-Vis) spectroscopy. Hydrogel discs (10 mm diameter) were immersed in phosphate-buffered saline (PBS) at 37 °C under constant agitation, and the release medium was completely collected and replaced with fresh PBS after 1, 2, 4, 6, 24, and 48 h to obtain cumulative release profiles. Calibration curves were established for both bioactive agents in PBS, and polymer interference was evaluated and subtracted before quantification. Release profiles were expressed as the cumulative percentage of NZ or CN released over time.

The antioxidant activity of the released fractions was assessed using the DPPH radical scavenging assay. Aliquots collected during the release study were incubated with DPPH solution, and radical scavenging activity was determined spectrophotometrically using Trolox as the positive control. Results were expressed as the percentage of DPPH reduction at steady state.

Quantitative antibacterial activity was evaluated according to the ASTM E2149 shake-flask method against *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Samples were incubated with bacterial suspensions (10^5 CFUs/mL) for 1, 2, 4, 6, and 24 h, after which serial dilutions were plated on agar for colony counting. Antibacterial performance was expressed as the percentage of bacterial reduction relative to untreated controls.

Antibiofilm activity was investigated after 48 h of bacterial growth using complementary assays to evaluate both biofilm biomass and metabolic activity. Total biomass was quantified by the crystal violet assay, whereas bacterial metabolic activity was determined using the resazurin assay. Results were expressed relative to untreated bacterial controls.

Cytocompatibility was assessed by direct contact according to ISO 10993-5 using HaCaT keratinocytes cultured under standard conditions. Sterilized hydrogels were placed directly onto the cell monolayer, and biological response was evaluated after 24 and 48 h by measuring metabolic activity using the resazurin assay, cell mass by sulforhodamine B (SRB) staining, and cell proliferation by Hoechst 33342 staining. Untreated cells and Triton X-100-treated cells were used as positive and negative controls, respectively.

2.3. Statistical analysis

All experiments were performed in triplicate unless otherwise stated in the Experimental Section. Results are expressed as mean $\pm$ standard deviation (SD). Statistical analysis was carried out using GraphPad Prism 8.0 software (GraphPad Software Inc., USA). Data normality was assessed prior to analysis. Differences between groups were evaluated using one-way or two-way analysis of variance (ANOVA). Statistical significance was considered at $p < 0.05$.

3. Results and discussion

3.1. Characterization of bioactive agents

3.1.1. Antimicrobial activity and synergistic interaction

The antimicrobial potential of NZ and CN was initially evaluated individually against the four bacterial species most frequently associated with infected chronic wounds, namely *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Their interaction was subsequently investigated to determine whether co-delivery could enhance antibacterial efficacy. The MIC and MBC values obtained for each bioactive agent are summarized in Table 2.

NZ exhibited pronounced antibacterial activity against the Gram-positive bacteria, with MIC and MBC values of 8 $\mu\text{g/mL}$ for both *S. aureus* and *S. epidermidis*. Conversely, substantially higher concentrations were required against the Gram-negative bacteria, particularly *E. coli*, for which the MBC reached 128 $\mu\text{g/mL}$. This susceptibility profile is consistent with the well-established mechanism of action of NZ, which involves binding to Lipid II, a key precursor of bacterial cell wall biosynthesis, thereby inhibiting peptidoglycan synthesis while simultaneously promoting pore formation in the cytoplasmic membrane. The resulting disruption of membrane integrity ultimately leads to bacterial cell lysis and explains the high efficacy of this class I bacteriocin against Gram-positive bacteria [32]. The reduced susceptibility observed for Gram-negative bacteria is mainly attributed to the presence of an outer membrane that acts as a permeability barrier, limiting peptide penetration and access to its intracellular targets [29].

CN displayed a similar susceptibility profile, although considerably higher bactericidal concentrations were required, particularly against Gram-negative bacteria. MIC values of 4 and 20 $\mu\text{g/mL}$ were obtained for *S. aureus* and *S. epidermidis*, respectively, whereas MBC values increased to 320 $\mu\text{g/mL}$ for *E. coli* and 160 $\mu\text{g/mL}$ for *P. aeruginosa*. The antibacterial activity of CN has been associated with disruption of the cytoplasmic membrane, increasing membrane permeability and promoting leakage of intracellular constituents [41]. Additionally, CN interferes with essential cellular processes, including DNA, RNA, and protein synthesis, ultimately impairing cell division and bacterial

Table 2

MIC and MBC determinations for NZ and CN against the four most prevalent bacteria in chronic wounds.

Active Agents	<i>S. aureus</i> ($\mu\text{g/mL}$)		<i>S. epidermidis</i> ($\mu\text{g/mL}$)		<i>E. coli</i> ($\mu\text{g/mL}$)		<i>P. aeruginosa</i> ($\mu\text{g/mL}$)	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
NZ	8	8	8	8	64	128	32	32
CN	4	80	20	20	320	320	80	160

survival [32,42,43]. As observed for NZ, the lower susceptibility of Gram-negative bacteria can be explained by the structural complexity of their outer membrane and their intrinsic resistance mechanisms.

Considering that the proposed hydrogel was designed for the simultaneous delivery of both bioactive agents, their antibacterial interaction was subsequently evaluated using the checkerboard assay. A pronounced synergistic effect was observed against the Gram-positive strains, with fractional inhibitory concentration index (FICI) values of 0.019 and 0.258 for *S. aureus* and *S. epidermidis*, respectively. These results demonstrate that combining NZ and CN substantially enhances antibacterial efficacy beyond that achieved by either compound alone, most likely because their complementary mechanisms of action facilitate bacterial membrane disruption while simultaneously interfering with essential intracellular processes [32,41]. Similar synergistic behavior has also been reported for *S. epidermidis* [44]. In contrast, the interaction became less favorable for the Gram-negative bacteria. An almost synergistic (additive) effect was observed for *E. coli* (FICI = 0.503), whereas an indifferent interaction was obtained for *P. aeruginosa* (FICI = 2.0). This behavior is consistent with previous reports and is primarily attributed to the intrinsic resistance of Gram-negative bacteria, particularly the presence of an outer membrane that restricts the penetration of both antimicrobial peptides and hydrophobic compounds, thereby limiting the benefits of combination therapy [45].

These findings demonstrate that NZ and CN provide complementary antibacterial functions. Although both compounds were intrinsically more effective against Gram-positive bacteria, their combination significantly enhanced antibacterial efficacy while extending antimicrobial coverage through the selection of concentrations corresponding to their individual MBC values. Consequently, 128 µg/mL of NZ and 320 µg/mL of CN were selected for subsequent hydrogel functionalization. Notably, most SA-based dressings reported to date incorporate a single antimicrobial agent, either a peptide or a plant-derived compound, rather than exploiting their combined mechanisms of action. By pairing a membrane-active, cell-wall-targeting bacteriocin with a broader-spectrum aldehyde, the present system extends antibacterial coverage beyond what either agent achieves alone, addressing a combination strategy that remains largely unexplored in fiber-reinforced SA wound dressings.

3.1.2. Phenolic content and antioxidant activity of the selected bioactive agents

Considering that oxidative stress is a major factor contributing to the chronic inflammatory environment and delayed healing of pressure ulcers, the phenolic content and antioxidant activity of the selected

bioactive agents were investigated to assess their potential contribution to the multifunctional hydrogel. The results are presented in Fig. 1.

CN exhibited significantly higher total phenolic content (TPC) than NZ (Fig. 1a), reaching approximately 130 mg GAE/g at 1 mg/mL ($p < 0.0001$), which is consistent with its aromatic structure and its classification as an intrinsic source of phenolic compounds [46,47]. When evaluated at its MBC concentration (320 µg/mL), TPC decreased markedly to approximately 26.7 mg GAE/g, confirming the expected concentration-dependent response resulting from the lower amount of phenolic compound available for reaction. Conversely, NZ exhibited an unexpected concentration-dependent behavior. At 1 mg/mL, only low TPC values were detected (≈ 10.8 mg GAE/g), whereas a marked increase (≈ 97.5 mg GAE/g) was observed at its MBC concentration (128 µg/mL). Because NZ is a lantibiotic peptide devoid of phenolic groups, this apparent increase is unlikely to represent genuine phenolic content. Instead, it is probably associated with the non-specific nature of the Folin-Ciocalteu assay, which is known to react with reducing compounds other than phenolics, together with possible molecular aggregation phenomena that may reduce the accessibility of reactive groups at higher concentrations [48].

The antioxidant activity determined by the DPPH assay (Fig. 1b) closely reflected the TPC results. CN exhibited pronounced radical scavenging activity, reaching values close to, or slightly exceeding, those obtained for the positive control (Trolox) at 1 mg/mL, confirming its strong reducing capacity [46,47]. Although DPPH reductions above 100% should be interpreted cautiously because the intrinsic colour of CN may interfere with absorbance measurements, the overall antioxidant profile clearly demonstrates its high radical scavenging potential. At its MBC concentration (320 µg/mL), antioxidant activity decreased to approximately 15%, mirroring the reduction in TPC and confirming the concentration-dependent behavior previously reported for phenolic compounds [47,49]. In contrast, NZ exhibited negligible antioxidant activity at both evaluated concentrations, with DPPH reduction remaining close to baseline throughout the 120 min assay. This behavior is consistent with its peptide structure, which lacks conjugated aromatic systems or functional groups capable of donating electrons or hydrogen atoms to stabilize free radicals [48]. Moreover, the absence of antioxidant activity supports the hypothesis that the apparent TPC detected for NZ results from methodological interference rather than from intrinsic phenolic properties.

The antioxidant response remained stable throughout the 120 min evaluation period for all tested conditions, indicating that radical scavenging occurred rapidly and was maintained over time [49]. This complementary behavior is particularly advantageous for chronic

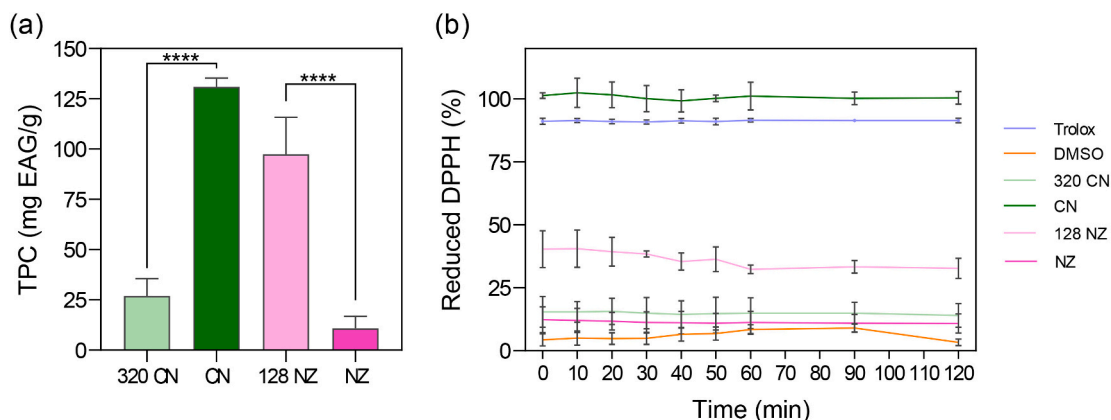


Fig. 1. (a) Total phenolic content (TPC), expressed as gallic acid equivalents (GAE), of CN and NZ evaluated at 1 mg/mL and at their respective minimum bactericidal concentrations (MBCs; 320 µg/mL for CN and 128 µg/mL for NZ). (b) DPPH radical scavenging activity of the same samples over 120 min. Trolox and DMSO were used as positive and negative controls, respectively. Data are presented as mean $\pm$ SD ($n = 3$). Statistical significance in (a) was determined using Sidak's multiple comparisons test, with **** indicating $p < 0.0001$. Statistical significance in (b) was determined using Tukey's multiple comparisons test, with Trolox differing significantly from DMSO, 320 CN, CN, 128 NZ, and NZ at all evaluated time points (**** $p < 0.0001$).

wound management, where simultaneous control of bacterial infection and oxidative stress is required to promote tissue repair. These findings demonstrate that CN is the main contributor to the antioxidant functionality of the proposed system, whereas NZ primarily provides antibacterial activity. Together with the synergistic antimicrobial interaction demonstrated in Section 3.1.1, these complementary biological properties further support the co-incorporation of both bioactive agents into the fiber-reinforced multifunctional hydrogel for chronic wound management.

3.2. Fragmentation of PCL electrospun fibers and modification with CN

Electrospinning successfully produced continuous and homogeneous PCL microfibers, which were subsequently converted into fragmented structures by alkaline hydrolysis (Fig. 2). The as-produced fibers exhibited smooth, defect-free surfaces and a high degree of uniformity, with an average diameter of $1.03 \pm 0.28 \mu\text{m}$. This morphology is consistent with the rheological and electrical properties of the electrospinning solution, which presented a high viscosity ($1196.8 \pm 8.7 \text{ mPa}\cdot\text{s}$) and low conductivity ($0.349 \pm 0.023 \mu\text{S}/\text{cm}$), conditions known to favor the formation of stable polymer jets and continuous fibers during electrospinning [24].

Following alkaline hydrolysis, the fibrous architecture was extensively modified, resulting in fragmented structures with rough and irregular surfaces. These morphological changes are characteristic of ester bond cleavage in PCL under alkaline conditions, leading to the formation of terminal hydroxyl and carboxyl groups and increasing surface roughness [50]. Besides reducing fiber dimensions, hydrolysis introduced terminal hydroxyl and carboxyl groups while increasing surface roughness, thereby generating a more reactive surface expected to facilitate subsequent CN immobilization.

To functionalize the reinforcement, PCL films and fragments were incubated with CN solutions ranging from the MBC to $100 \times \text{MBC}$. Because of the small size of the fragments and adsorption losses during filtration, quantitative analysis could only be reliably performed for the PCL films. CN immobilization increased with increasing solution concentration up to $16.00 \text{ mg}/\text{mL}$, after which a decrease was observed, suggesting saturation or partial desorption of the compound at higher concentrations. Complete loading data are presented in Table S1 (Section S2 of Supporting Information).

Based on these results, an initial CN concentration of $6.40 \text{ mg}/\text{mL}$ ($20 \times \text{MBC}$) was selected for subsequent experiments, as it provided a functionalized amount well above the MBC required for antibacterial activity while remaining below the concentration range associated with saturation and partial desorption of the compound. This condition yielded a functionalized amount of $2.793 \pm 0.357 \text{ mg}/\text{mL}$, well above the MBC required for antibacterial activity (Section 3.1.1). Although CN loading could not be quantified directly for the fragmented fibers, their disrupted and irregular morphology, resulting from alkaline hydrolysis

(Fig. 2), is expected to favor CN retention through two complementary mechanisms. First, the increased surface roughness and reduced fragment dimensions substantially increase the surface-area-to-volume ratio available for compound adsorption, compared to the smooth, continuous architecture of the intact PCL film. Second, the predominantly hydrophobic character of both PCL and CN favors their mutual affinity, so that the irregular surface generated by hydrolysis, together with the partially exposed internal fiber structure, likely provides additional retention sites through hydrophobic interactions and physical entrapment within surface irregularities, beyond simple superficial adsorption. This structural rationale supports the use of fragmented fibers as reinforcing elements within the hydrogel matrix. The results demonstrate that alkaline hydrolysis provides an effective strategy for generating a fibrous reinforcement with enhanced surface characteristics for CN immobilization. The selected functionalization conditions ensured bactericidal amounts of immobilized CN while preserving the structural features required for incorporation into the multifunctional hydrogel.

3.3. Development and functionalization of the reinforced hydrogel

Following optimization of the fabrication process, three hydrogel formulations were successfully produced: unmodified sodium alginate hydrogels (SA), hydrogels reinforced with intact PCL films (SA/FL), and hydrogels reinforced with fragmented PCL fibers (SA/FR). Representative macroscopic images are presented in the Supporting Information (Fig. S1, Section S2–2.2). All formulations exhibited homogeneous surfaces without visible defects, confirming the robustness of the fabrication method. Slight heterogeneity was observed in the SA/FR hydrogels owing to the more challenging dispersion of the fragmented fibers within the polymer matrix, despite the application of magnetic stirring and ultrasonic treatment. Peripheral regions containing excess material resulting from the molding process were excluded from all subsequent analyses to ensure uniform sample thickness.

The internal morphology of the hydrogels was further evaluated by SEM (Fig. 3). Representative micrographs confirmed the successful incorporation of the reinforcing structures into the SA matrix. In SA/FL hydrogels, the PCL film remained clearly distinguishable, preserving its fibrous architecture within the hydrogel. In contrast, SA/FR hydrogels exhibited localized clusters of fragmented fibers, corroborating the slight dispersion limitations observed macroscopically (Fig. S1, Section S2 from Supporting Information). Minor surface irregularities observed in the SA hydrogels were attributed to residual salts remaining after ionic crosslinking; however, these were effectively removed during the subsequent washing and sterilization procedures performed before biological evaluation.

To functionalize the hydrogel matrix, samples were incubated in NZ solutions ranging from the MBC to $100 \times \text{MBC}$. Since CN-functionalized fibers were expected to occupy part of the available binding sites, quantitative analysis was performed using SA hydrogels only. NZ

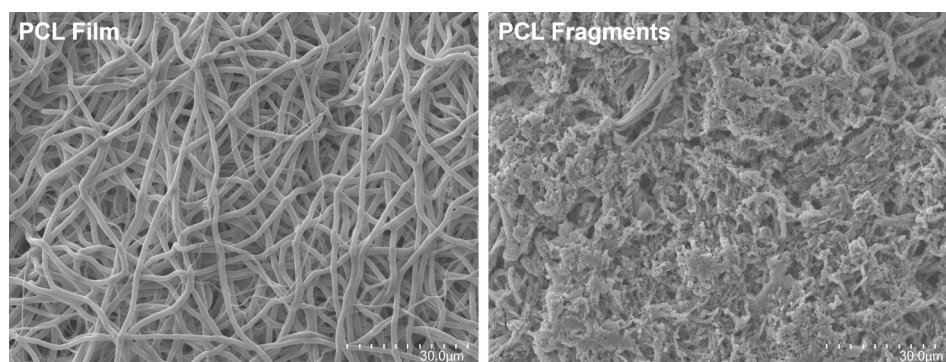


Fig. 2. SEM micrographs of electrospun PCL structures: (left) as-produced PCL film showing continuous and uniform fibers; (right) hydrolyzed PCL fragments exhibiting disrupted morphology, with partially preserved fibrous structures (scale bar: $30 \mu\text{m}$).

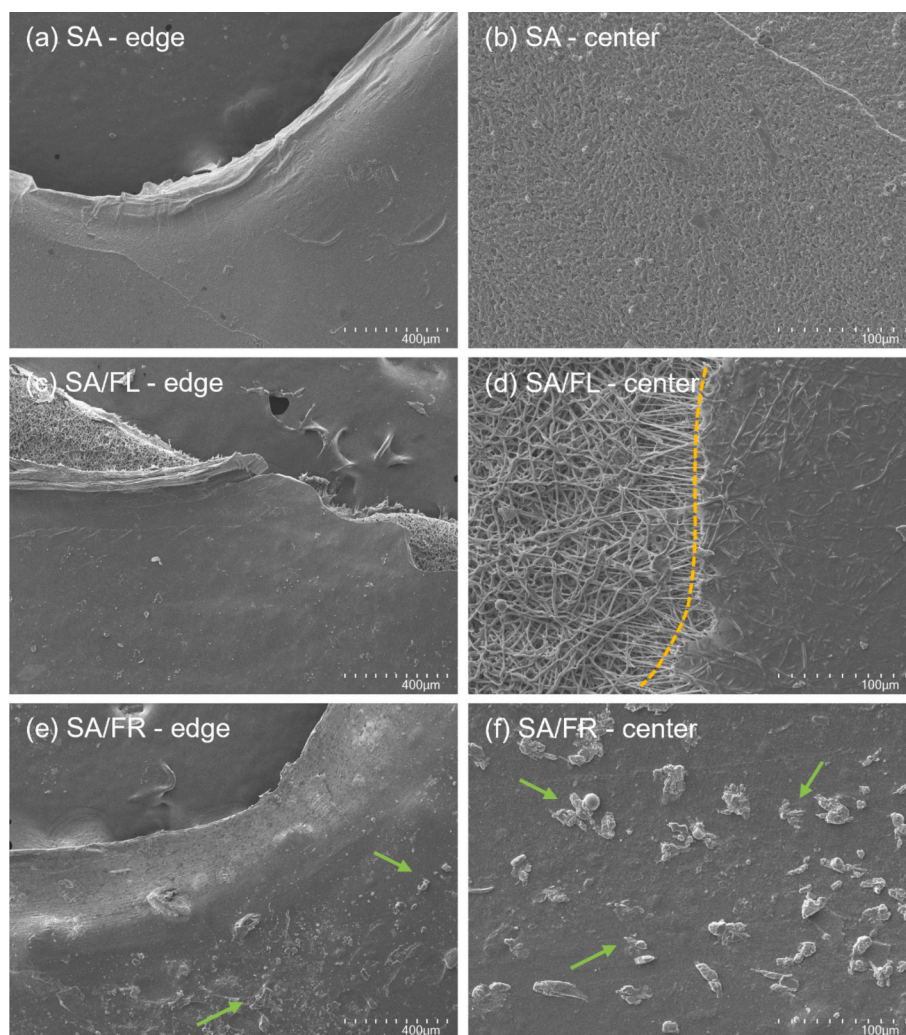


Fig. 3. SEM micrographs of hydrogels: (a,b) SA, (c,d) SA/FL, and (e,f) SA/FR (green arrows highlight some of the fragmented fibers). Images on the left correspond to the edge/cross-section region of the hydrogels (scale bar: 400 μm), while images on the right correspond to a more central region (scale bar: 100 μm). The surface of sample (d) was purposely damaged to observe the fiber film underneath (damaged area on the left separated by dashed yellow line).

incorporation increased progressively with the initial solution concentration, demonstrating concentration-dependent loading within the alginate network. Complete NZ loading data are presented in Table S2 (Supporting Information, Section S2). Based on these results and considering the MBC values established in Section 3.1.1, an initial NZ concentration of 2.560 mg/mL ($20 \times \text{MBC}$) was selected for subsequent experiments, yielding a functionalized amount of 0.286 ± 0.082 mg/mL, which exceeded the bactericidal threshold required against all tested bacterial strains. Although bactericidal loading could also be achieved using 1.280 mg/mL ($10 \times \text{MBC}$), this condition exhibited greater variability and values closer to the minimum effective concentration, potentially compromising reproducibility. Therefore, the $20 \times \text{MBC}$ condition was selected to ensure more consistent antibacterial performance.

Data demonstrated that SA hydrogels can be successfully reinforced with either intact PCL films or fragmented fibers while simultaneously incorporating bactericidal amounts of NZ without compromising the structural integrity of the hydrogel. Combined with the previously developed CN-functionalized fibrous reinforcement, this strategy enabled the fabrication of a fiber-reinforced multifunctional hydrogel integrating complementary antibacterial and antioxidant functionalities, thereby providing a promising platform for chronic wound management.

3.4. Chemical, thermal, and mechanical characterization

3.4.1. Chemical analysis (ATR-FTIR)

ATR-FTIR spectroscopy was used to evaluate the chemical composition of the developed systems and to confirm the successful incorporation of the different components into the hydrogels. The spectra of the raw materials, intermediate structures, and final hydrogels are presented in Fig. S2 (Section S3 from Supporting Information). The characteristic absorption bands observed for SA, PCL, NZ, and CN agreed with those previously reported in the literature, confirming the chemical identity of all components [32,51,52] [53].

The control SA hydrogel retained the characteristic alginate absorption bands, indicating that ionic crosslinking did not induce significant chemical modifications to the polymer matrix. Likewise, the spectra of SA + NZ hydrogels exhibited the characteristic amide I and amide II bands of NZ, confirming successful peptide incorporation and retention after the washing procedure. Similarly, SA/FL and SA/FR hydrogels displayed the characteristic bands of both SA and PCL, demonstrating successful incorporation of the fibrous reinforcement into the hydrogel matrix.

In the functionalized systems, partial overlap of characteristic absorption bands was observed because of the coexistence of multiple components. In particular, PCL-related bands were less pronounced in FR-containing hydrogels, most likely owing to the smaller fragment size,

their heterogeneous distribution within the matrix, and signal masking by the dominant alginate network. Likewise, in CN-containing systems, identification of some PCL bands became more difficult because of spectral overlap with the characteristic absorptions of CN and the surface-sensitive nature of ATR-FTIR analysis. Nevertheless, the coexistence of the characteristic bands of SA, PCL, NZ, and CN confirmed the successful incorporation of all constituents into the multifunctional hydrogel. The presence of the fibrous reinforcement was further corroborated by the SEM observations (Fig. 3).

Overall, ATR-FTIR analysis demonstrated that neither hydrogel fabrication nor bioactive functionalization altered the chemical identity of the individual components, while confirming the successful assembly of the proposed fiber-reinforced multifunctional hydrogel.

3.4.2. Thermal analysis

Thermogravimetric analysis (TGA), derivative thermogravimetry (DTG), and differential scanning calorimetry (DSC) were performed to evaluate the thermal stability of the individual materials, PCL-based structures, and developed hydrogels, as well as to investigate potential interactions between the different constituents.

The thermal behavior of the individual components (SA, NZ, PCL, and CN) and PCL-based structures (FL, FR, FL + CN, and FR + CN) is presented in Section S4 (Fig. S3 and Table S3, Supporting Information). Overall, the obtained thermal profiles were consistent with those previously reported in the literature [26,54]. SA exhibited the lowest thermal stability owing to its hygroscopic polysaccharide structure, whereas PCL showed the highest thermal stability, with a characteristic melting transition followed by degradation at higher temperatures [24]. NZ presented degradation events associated with peptide bond cleavage [24], while CN exhibited lower thermal stability due to its volatile aromatic aldehyde structure [26,54]. Following CN functionalization, slight shifts in degradation temperatures and modifications of the PCL melting transition were observed, particularly in the fragmented structures, suggesting interactions between CN and the PCL matrix that may be enhanced by the higher specific surface area generated after hydrolysis.

The thermal behavior of the hydrogel formulations is presented in Section S4 (Fig. S4 and Table S4, Supporting Information). All hydrogels exhibited an initial mass loss below approximately 150 °C, associated with water evaporation from the alginate matrix [55]. Incorporation of the PCL reinforcement resulted in broader and slightly shifted melting transitions, suggesting reduced crystallinity owing to interactions

between PCL and the alginate network [56,57]. Likewise, incorporation of NZ promoted attenuation of the characteristic peptide thermal transitions together with slight shifts in degradation temperatures, indicating partial encapsulation and thermal stabilization within the hydrogel matrix. Hydrogels containing CN also exhibited attenuation of CN-related thermal events, suggesting reduced volatilization and stronger interactions between the bioactive compound and the surrounding polymeric network [58].

In the fully functionalized hydrogels, overlapping thermal events reflected the coexistence of SA, PCL, NZ, and CN. Nevertheless, despite the intrinsic thermal instability of NZ and CN, the presence of PCL delayed the overall degradation process, acting as a stabilizing structural component [59]. Generally, the thermal analysis demonstrated that incorporation of both bioactive agents and the fibrous reinforcement did not compromise the thermal stability of the developed system, while the observed shifts and attenuation of characteristic thermal events further support the successful integration and interaction of all components within the proposed fiber-reinforced multifunctional hydrogel.

3.4.3. Disintegration, hydration, porosity, and air permeability

The disintegration profile, degree of hydration, porosity, and air permeability of the developed hydrogels are summarized in Table 3. Together, these parameters provide a comprehensive assessment of the structural stability and transport properties of the materials under physiological conditions, which are critical for wound dressings intended to maintain a moist environment while allowing oxygen diffusion and controlled degradation.

The control SA hydrogel exhibited progressive disintegration throughout the 7-day incubation period, reaching $47.38 \pm 12.38\%$ mass loss after 4 days and undergoing complete disintegration by day 7. This behavior reflects the limited long-term stability of ionically crosslinked alginate hydrogels, which gradually lose structural integrity because of ion exchange with the surrounding medium and relaxation of the polymeric network [24]. Although such behavior is characteristic of alginate, controlled degradation is desirable for wound dressings because it facilitates gradual replacement of the material by newly formed tissue. The incorporation of PCL significantly modified the disintegration profile, although the effect strongly depended on the morphology of the reinforcement. Hydrogels reinforced with continuous PCL films (SA/FL) exhibited higher apparent mass loss throughout the incubation period, reaching $81.67 \pm 3.43\%$ after 4 days. This behavior is likely associated with partial delamination between the hydrophobic

Table 3

Disintegration profile, degree of hydration, porosity, and air permeability of SA-based hydrogels. Disintegration was evaluated as mass loss (%) over 7 days of incubation in PBS at 37 °C. Degree of hydration was determined after 24 and 48 h of incubation in PBS at 37 °C. Porosity was measured after 1 h immersion in ethanol, and air permeability was evaluated according to ISO 9237 under a pressure of 200 Pa. Data are presented as mean $\pm$ SD ($n = 3$).

Samples	Disintegration Profile - Mass loss (%)					Degree of Hydration (%)		Porosity (%)	Air permeability (L/min)
	1st day	2nd day	3rd day	4th day	7th day	24 h	48 h		
SA	17.06 $\pm$ 6.18	25.83 $\pm$ 2.71	35.19 $\pm$ 8.32	47.38 $\pm$ 12.38	–	98.81 $\pm$ 0.47	98.54 $\pm$ 0.41	0.421 $\pm$ 0.104	0.024 $\pm$ 0.006
SA /FL	35.30 $\pm$ 2.87	42.85 $\pm$ 7.82	54.40 $\pm$ 4.54	81.67 $\pm$ 3.43	92.79 $\pm$ 0.06	95.49 $\pm$ 1.46	68.41 $\pm$ 22.30	5.100 $\pm$ 1.587	0.011 $\pm$ 0.005
SA /FR	19.29 $\pm$ 11.87	15.43 $\pm$ 11.58	24.22 $\pm$ 16.91	33.19 $\pm$ 16.91	–	94.20 $\pm$ 0.90	–	14.310 $\pm$ 3.765	0.024 $\pm$ 0.007
SA + NZ	41.22 $\pm$ 9.38	42.12 $\pm$ 13.15	45.44 $\pm$ 12.09	67.06 $\pm$ 11.32	–	92.93 $\pm$ 3.95	91.29 $\pm$ 3.40	9.907 $\pm$ 7.063	0.033 $\pm$ 0.008
SA + NZ/FL	87.62 $\pm$ 2.33	85.94 $\pm$ 7.11	88.06 $\pm$ 4.08	87.91 $\pm$ 4.73	90.94 $\pm$ 2.95	97.10 $\pm$ 0.34	91.13 $\pm$ 5.43	13.691 $\pm$ 5.250	0.003 $\pm$ 0.001
SA + NZ/FR	43.43 $\pm$ 5.31	54.14 $\pm$ 10.63	65.89 $\pm$ 6.48	67.57 $\pm$ 3.99	–	94.31 $\pm$ 0.55	–	6.864 $\pm$ 5.139	0.003 $\pm$ 0.002
SA/FL + CN	16.99 $\pm$ 5.36	50.41 $\pm$ 31.54	73.45 $\pm$ 22.67	91.28 $\pm$ 6.72	94.12 $\pm$ 1.05	96.90 $\pm$ 1.13	75.82 $\pm$ 14.70	2.483 $\pm$ 1.052	0.000 $\pm$ 0.000
SA/FR + CN	22.49 $\pm$ 11.79	18.64 $\pm$ 4.15	27.52 $\pm$ 2.70	–	–	97.26 $\pm$ 0.74	–	1.609 $\pm$ 0.725	0.007 $\pm$ 0.000
SA + NZ/FL + CN	21.56 $\pm$ 11.84	18.41 $\pm$ 39.35	19.40 $\pm$ 32.57	16.30 $\pm$ 13.25	22.48 $\pm$ 29.14	95.93 $\pm$ 1.17	88.01 $\pm$ 4.32	5.281 $\pm$ 4.657	0.000 $\pm$ 0.000
SA + NZ/FR + CN	14.93 $\pm$ 5.90	–	–	–	–	97.55 $\pm$ 1.20	–	0.5991 $\pm$ 0.090	0.000 $\pm$ 0.000

PCL film and the hydrophilic alginate matrix, creating preferential pathways for PBS penetration and accelerating hydrogel erosion. Nevertheless, residual PCL structures remained visible after 7 days, confirming that the observed mass loss mainly reflected degradation of the alginate matrix rather than the polyester reinforcement itself. In contrast, hydrogels reinforced with fragmented fibers (SA/FR) displayed a slower initial degradation profile, with only $33.19 \pm 16.91\%$ mass loss after 4 days. This suggests that the fragmented morphology promotes more homogeneous dispersion throughout the hydrogel and improves mechanical interlocking between the fibers and the alginate network, temporarily delaying structural disintegration. However, complete degradation still occurred before day 7, indicating that this reinforcing effect is primarily beneficial during the early stages of incubation. The bioactive agents further influenced hydrogel stability. NZ alone produced only moderate changes in the degradation profile, indicating that peptide incorporation does not substantially alter the structural organization of the alginate matrix. Conversely, CN exerted a markedly different effect, particularly in fragment-reinforced systems. Hydrogels containing CN exhibited accelerated degradation, with SA/FR + CN undergoing complete disintegration by day 4. This response is probably related to the hydrophobic aromatic structure of CN, which may disturb the hydrophilic alginate network, reduce intermolecular cohesion, weaken fiber-matrix interactions, and promote the formation of microstructural defects [60]. Interestingly, the fully functionalized SA + NZ/FL + CN hydrogel showed the highest structural stability among all formulations, exhibiting only $22.48 \pm 29.14\%$ mass loss after 7 days. This finding suggests that NZ may partially compensate for the destabilizing effect of CN by promoting additional intermolecular interactions, such as hydrogen bonding, thereby reinforcing the polymeric network and reducing chain mobility.

The hydration study complemented the degradation results and further highlighted the influence of both reinforcement morphology and bioactive incorporation (Table 3). All formulations exhibited hydration values exceeding 90% after 24 h, confirming the excellent water-retention capacity of alginate-based hydrogels. Such high hydration is advantageous for chronic wound treatment because it contributes to maintaining a moist microenvironment, facilitates nutrient diffusion, promotes autolytic debridement, and assists exudate management. More pronounced differences became evident after 48 h. Hydrogels containing continuous PCL films exhibited a substantial reduction in hydration, decreasing from $95.49 \pm 1.46\%$ to $68.41 \pm 22.30\%$, reflecting the hydrophobic nature of PCL and its tendency to reduce water diffusion through the polymeric network. Fragment-reinforced hydrogels maintained considerably higher hydration, suggesting that the discontinuous morphology of the fibers allows better integration within the alginate matrix while preserving its hydrophilic character. NZ-containing hydrogels also maintained hydration levels comparable to the control throughout the evaluation period, indicating that the peptide does not compromise water retention. In contrast, incorporation of CN promoted a reduction in hydration after prolonged incubation, particularly in film-reinforced hydrogels, which may be attributed to the hydrophobic character of the aromatic aldehyde and the resulting microstructural rearrangements within the polymer network [26,61]. Interestingly, when NZ and CN were simultaneously incorporated, hydration remained relatively high, suggesting that NZ partially offsets the hydrophobic effects introduced by CN and contributes to preserving the hydrated hydrogel structure.

The internal architecture of the hydrogels was further assessed through porosity and air permeability measurements. The control SA hydrogel exhibited low porosity and limited air permeability, consistent with the formation of a relatively dense alginate network in which transport occurs mainly through the hydrated polymer matrix rather than through interconnected pores. Incorporation of PCL significantly modified these properties according to the reinforcement morphology. Continuous PCL films increased porosity but simultaneously reduced air permeability, indicating that the newly generated voids were poorly

interconnected and therefore did not facilitate gas transport. Conversely, fragmented fibers produced the highest porosity values while maintaining permeability similar to the control hydrogel, supporting the hypothesis that they act as spacers within the polymeric matrix and generate a more homogeneous porous architecture. The bioactive agents also affected the internal microstructure. NZ increased porosity and slightly enhanced air permeability, suggesting that peptide incorporation promotes a less compact polymeric arrangement without compromising network integrity. However, when combined with PCL reinforcement, permeability decreased markedly despite the increase in porosity, indicating that pore connectivity rather than pore volume governs gas transport through these systems. Hydrogels containing CN exhibited the lowest permeability values together with reduced porosity, suggesting that CN promotes structural rearrangement and partial blockage of transport pathways because of its hydrophobic interactions with the surrounding matrix. Consequently, although porosity is generally associated with improved permeability, the present results demonstrate that the connectivity and continuity of the pore network are more important determinants of transport behavior than porosity alone. This interpretation is further supported by the SEM observations (Fig. 3), which revealed heterogeneous internal structures and limited pore interconnectivity in CN-containing hydrogels. Notably, this pattern is consistent with the hydration results discussed above: the poorly interconnected voids generated within PCL films likely restrict water diffusion despite their higher porosity, whereas the more homogeneous and interconnected porosity produced by fragmented fibers facilitates water uptake, explaining the higher hydration retained by SA/FR hydrogels compared to SA/FL.

Data demonstrates that both the morphology of the PCL reinforcement and the combination of NZ and CN provide effective tools for tailoring hydrogel degradation, hydration, and transport properties. Continuous PCL films improve long-term structural integrity, fragmented fibers generate more porous architectures, CN accelerates degradation while reducing permeability, and NZ contributes to preserving hydration and partially counteracts the destabilizing effects of CN. Such complementary effects are particularly advantageous for chronic wound dressings, where balancing structural stability, moisture retention, oxygen transport, and controlled degradation is essential for supporting tissue regeneration.

3.4.4. Mechanical evaluations

The mechanical performance of the hydrogels was evaluated in terms of adhesiveness, compressive strength, and recovery capacity (Fig. 4), as these properties determine the ability of a wound dressing to remain attached to the wound bed while accommodating the mechanical stresses associated with patient movement.

The adhesion of the hydrogels was evaluated using tracing paper as a skin model, and the detachment force was measured after applying a standardized load (Fig. 4a). The control SA hydrogel exhibited the lowest adhesion strength, which can be attributed to its homogeneous and highly hydrated structure. The presence of water at the interface promotes a lubricating effect, reducing friction and limiting adhesion [62]. The incorporation of PCL, either as film (SA/FL) or fragments (SA/FR), did not significantly alter adhesion, indicating that PCL alone does not contribute meaningfully to interfacial bonding. In contrast, the incorporation of NZ (SA + NZ) resulted in a significant increase in adhesion ($p < 0.0001$). This behavior can be attributed to the amphipathic nature of the peptide, which promotes surface reorganization and enhances moisture retention, increasing conformability and contact with the substrate [29]. These effects facilitate interfacial interactions and improve adhesion. However, when NZ was combined with PCL (SA + NZ/FL and SA + NZ/FR), adhesiveness returned to levels comparable to the control. This suggests that the presence of PCL limits the exposure and effectiveness of NZ at the interface, reducing its ability to enhance adhesion.

Hydrogels containing CN (SA/FL + CN and SA/FR + CN) exhibited

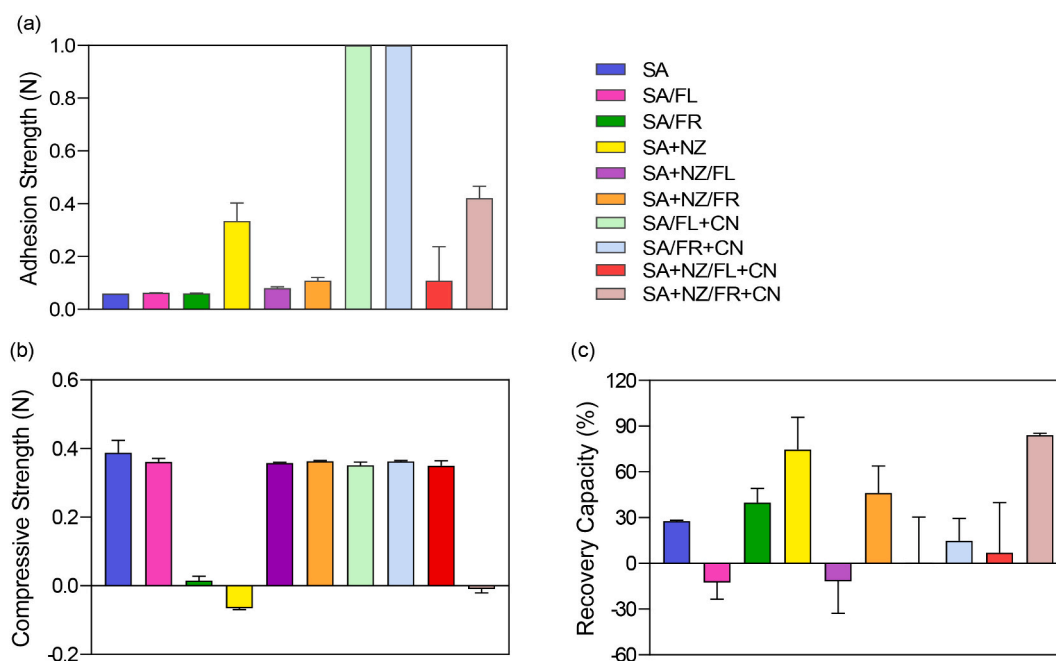


Fig. 4. Mechanical performance of SA-based hydrogels: (a) adhesion strength measured to paper using a detachment test after applying a 1.5 kg load for 5 min; (b) compressive strength determined at maximum force under compression at 1 mm/min; and (c) recovery capacity (%) after three compression cycles at 50% deformation. For SA/FL + CN and SA/FR + CN samples, detachment was not achieved, and values were recorded as the maximum measurable force. Data are presented as mean $\pm$ SD ($n = 3$). Statistical significance was assessed using Dunnett's multiple comparisons test with the control sample SA, with significance being detected for: (a) $***p < 0.0001$ for SA vs. SA + NZ, SA/FL + CN, SA/FR + CN and SA + NZ/FR + CN; (b) $***p < 0.0001$ for SA vs. SA/FR, SA + NZ and SA + NZ/FR + CN, $*p < 0.05$ for SA vs. SA/FL + CN and SA + NZ/FL + CN; (c) $*p < 0.05$ for SA vs. SA + NZ and SA + NZ/FR + CN.

the highest adhesion, reaching the maximum measurable force ($p < 0.0001$). This behavior can be explained by the plasticizing effect of CN, which increases matrix flexibility and promotes better surface contact, as well as by the presence of reactive aldehyde groups that enhance intermolecular interactions with the substrate [63]. Additionally, CN may increase chain mobility at the interface, improving wetting and adhesion. When NZ and CN were combined, adhesion decreased compared to CN-only systems. Although SA + NZ/FR + CN still showed significantly higher adhesion, the reduction observed suggests competition between NZ and CN at the interface. NZ may promote a smoother and more hydrated surface, which can partially hinder CN-driven interactions, reducing the overall adhesive effect. The results show that CN acts as the primary adhesion enhancer, whereas NZ mainly modulates the interfacial properties of the hydrogel. Such balance is particularly important for wound dressings, since excessive adhesion may damage newly formed tissue during removal, whereas insufficient adhesion compromises dressing fixation.

The compressive strength and recovery capacity of the hydrogels were subsequently evaluated after three compression cycles at 50% deformation (Fig. 4b and c). SA hydrogel exhibited moderate compressive strength (≈ 0.39 N) and limited recovery ($\approx 30\%$), reflecting a balance between structural integrity and elasticity. The incorporation of PCL film (SA/FL) did not significantly affect compressive strength but reduced recovery, indicating increased rigidity and reduced ability to recover after deformation. This suggests that the continuous hydrophobic phase restricts polymer chain mobility. In contrast, SA/FR exhibited a significant reduction in compressive strength ($p < 0.0001$), indicating weakened structural cohesion. However, this system showed improved recovery, suggesting that fragmented fibers promote elasticity by enabling localized deformation and structural rearrangement within the matrix.

The incorporation of NZ (SA + NZ) led to a significant decrease in compressive strength, indicating reduced resistance to applied stress. However, recovery capacity increased significantly, suggesting that NZ enhances flexibility and resilience. This effect can be attributed to its

amphipathic nature, which increases water retention and facilitates polymer chain mobility, promoting elastic recovery at the expense of mechanical strength [29]. When NZ was combined with PCL film (SA + NZ/FL), its effect was minimized, with mechanical behavior similar to SA/FL. This indicates that the film structure limits NZ interaction with the hydrogel network. On the other hand, SA + NZ/FR showed improved mechanical performance compared to SA/FR, suggesting that better dispersion of NZ enhances its ability to promote network reorganization and elasticity.

The incorporation of CN resulted in moderate compressive strength values. Significant increases were observed in SA/FL + CN, SA + NZ/FL + CN and SA + NZ/FR + CN. These results demonstrate that CN contributes to structural reinforcement, likely through a plasticizing effect that enhances intermolecular cohesion and load distribution within the matrix, even though recovery capacity was generally reduced in CN-containing systems, reflecting a potential increased rigidity and reduced chain mobility. Notably, SA + NZ/FR + CN exhibited a significant increase in recovery ($p < 0.05$), suggesting a synergistic effect between NZ and CN when both are well dispersed. In this system, NZ promotes flexibility, while CN maintains structural cohesion, resulting in a material that is both resilient and mechanically stable.

As observed, the mechanical performance of the hydrogels is governed by the interplay between rigidity and flexibility. PCL film increases stiffness but reduces elasticity, fragmented fibers promote recovery but weaken strength, NZ enhances flexibility and resilience, and CN improves mechanical resistance while limiting recovery. Their simultaneous incorporation, particularly in fragment-reinforced hydrogels, provides a favorable balance between adhesion, mechanical resistance, and deformability, which is highly desirable for wound dressings that must remain securely attached while adapting to repeated mechanical deformation during normal patient movement. These results reinforce that mechanical performance in SA-based dressings is typically achieved at the expense of flexibility, or vice versa, when a single reinforcement strategy is used. By offering two structurally distinct reinforcement options within the same bioactive-loaded matrix, the

present platform allows the mechanical profile of the dressing to be tuned to the specific demands of the wound site, a level of tunability not offered by conventional single-reinforcement alginate systems.

3.4.5. Biological assessments of the reinforced hydrogels

3.4.5.1. NZ and CN release kinetics. The release profiles of NZ and CN were evaluated by UV–Visible spectroscopy after incubation of the hydrogels in PBS at 37 °C under agitation (120 rpm) for up to 48 h (Fig. 5). Aliquots were collected at predefined time points (1, 2, 4, 6, 24, and 48 h), and the release medium was replenished with fresh PBS. Calibration curves were established for both NZ and CN to correlate absorbance with concentration. To ensure accuracy, release data were calculated based on the difference in absorbance between loaded and unloaded systems, eliminating potential contributions from polymeric compounds leached from the hydrogel matrix.

For NZ (Fig. 5a), all systems exhibited a biphasic release profile, characterized by an initial burst release followed by a slower, sustained release phase. The initial stage is attributed to the diffusion of NZ molecules weakly bound or located at the hydrogel surface. The subsequent phase is governed by diffusion through the polymer network and interactions between NZ and the matrix. The SA + NZ/FR system showed the highest cumulative release, which can be attributed to the increased porosity and structural heterogeneity introduced by the fragmented fibers. These features enhance water penetration and create diffusion pathways that facilitate NZ release. In contrast, SA + NZ/FL exhibited a more controlled and limited release, likely due to the presence of a continuous PCL film that acts as a diffusion barrier. Additionally, electrostatic interactions between the positively charged regions of NZ and the negatively charged SA matrix may contribute to partial retention of the peptide, particularly in more compact systems. Data shows that NZ release kinetics is strongly influenced by hydrogel morphology, with more open and heterogeneous structures promoting faster diffusion, while denser systems favor sustained release. This behavior is consistent with diffusion-controlled release mechanisms commonly observed in SA-based hydrogels [24].

CN release (Fig. 5b) was ten times lower than NZ and remained relatively constant over time. This behavior is primarily associated with the hydrophobic nature and low aqueous solubility of CN, which limits its diffusion into the PBS medium. Furthermore, CN is preferentially associated with PCL structures, which are also hydrophobic and act as reservoirs that retard release. Differences between systems were also observed. In SA/FL + CN, the continuous PCL film promotes a more gradual and controlled release, while in SA/FR + CN, the higher surface area of fragmented fibers allows for slightly increased release, likely due

to enhanced local exposure. However, overall release remains limited, suggesting strong retention of CN within the hydrophobic domains of the system. The presence of NZ did not alter the CN release profile, as observed in SA + NZ/FL + CN and SA + NZ/FR + CN systems. This indicates that both agents are released independently, with NZ governed by diffusion through the hydrophilic matrix and CN controlled by its hydrophobic interactions and low solubility. From an application perspective, this dual-release behavior is advantageous, as it allows rapid availability of NZ for immediate antimicrobial action, while CN remains retained within the system, potentially providing prolonged activity at the material interface.

3.4.5.2. Antioxidant activity. The antioxidant activity of the hydrogels was evaluated using the DPPH assay, based on the ability of the released compounds to scavenge free radicals. Hydrogels were incubated in PBS at 37 °C, and aliquots were collected at different time points (1 to 48 h). These aliquots were subsequently reacted with DPPH for up to 120 min, and the antioxidant activity was monitored over time (Fig. 6). Here, only CN-containing systems (SA/FL + CN, SA/FR + CN, SA + NZ/FL + CN, and SA + NZ/FR + CN) were evaluated, as NZ did not exhibit intrinsic antioxidant activity (Section 3.1.2). Thus, this section focuses exclusively on CN-driven antioxidant behavior, minimizing interference from the remainder components.

At early time points (1–2 h, Fig. 6a and b), only SA/FR + CN and SA + NZ/FL + CN exhibited positive antioxidant activity. At 1 h, SA/FR + CN and SA + NZ/FL + CN showed significantly higher activity compared to SA/FL + CN ($p < 0.0001$), confirming a faster availability of CN in these systems. This behavior is consistent with the release profiles (Fig. 5b), where these formulations showed higher initial CN release. In contrast, SA/FL + CN and SA + NZ/FR + CN exhibited values close to zero or slightly negative, indicating limited CN availability. The enhanced activity of SA/FR + CN can be attributed to the higher surface area of fragmented fibers, which promotes burst release. Similarly, SA + NZ/FL + CN showed early activity, suggesting that NZ facilitates CN diffusion, likely by increasing matrix hydration and mobility.

Between 4 and 6 h (Fig. 6c and d), significant differences between systems became more evident. At 4 h, SA/FL + CN remained significantly lower than all other systems, confirming its limited release capacity. At 6 h, SA + NZ/FR + CN also showed significantly lower activity compared to both SA/FR + CN and SA + NZ/FL + CN, despite showing a release peak in Fig. 5. This discrepancy suggests that not all released CN is effectively available for radical scavenging, possibly due to rapid oxidation, instability in aqueous media, or retention within microdomains of the matrix.

At longer time points (24 and 48 h, Fig. 6e and f), all systems showed

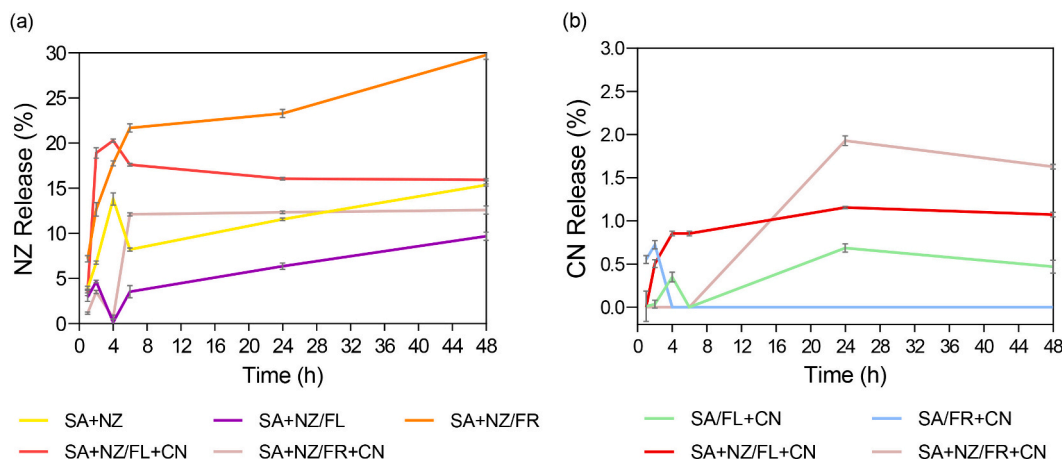


Fig. 5. Release profiles of the active agents (a) NZ and (b) CN from SA-based hydrogels after 1, 2, 4, 6, 24, and 48 h of incubation in PBS. Release values were corrected by subtracting the contribution of unloaded systems to account for potential polymer interference. Data are presented as mean $\pm$ SD ($n = 3$).

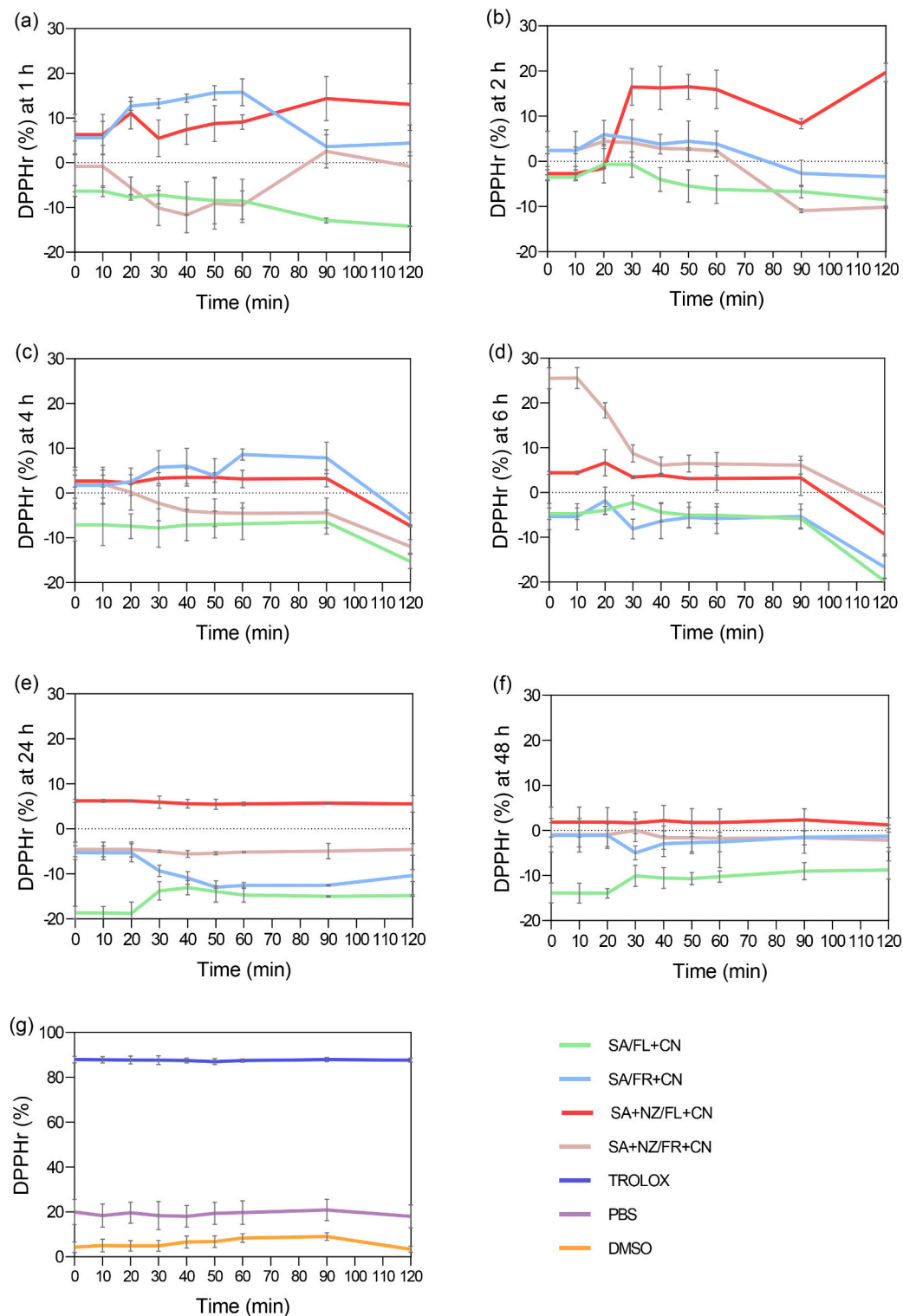


Fig. 6. Antioxidant activity of CN-containing hydrogels evaluated by the DPPH assay after (a) 1, (b) 2, (c) 4, (d) 6, (e) 24, and (f) 48 h of incubation in PBS, followed by up to 120 min of reaction with DPPH at 37 °C and 120 rpm; (g) control samples, namely Trolox (positive control), DMSO (negative control), and PBS (solvent). Data are presented as mean $\pm$ standard deviation (SD, $n = 3$). Statistical significance was assessed using Tukey's multiple comparisons test. Significant differences were observed depending on incubation time, namely: (a) $****p < 0.0001$ for all comparisons except SA/FL + CN vs. SA + NZ/FR + CN and SA/FR + CN vs. SA + NZ/FL + CN; (b) $***p < 0.001$ for SA/FL + CN vs. SA + NZ/FL + CN and $**p < 0.01$ for SA + NZ/FL + CN vs. SA + NZ/FR + CN; (c) $****p < 0.0001$ for most comparisons involving SA/FL + CN, with additional $**p < 0.01$ and $*p < 0.05$ differences between remaining systems; (d) $****p < 0.0001$ for comparisons involving SA + NZ/FR + CN, with $*p < 0.05$ for remaining significant pairs; (e) $****p < 0.0001$ for all comparisons except SA/FR + CN vs. SA + NZ/FR + CN ($***p < 0.001$); (f) $****p < 0.0001$ for all comparisons except SA/FR + CN vs. SA + NZ/FR + CN (non significant).

a decrease in antioxidant activity, consistent with depletion of available CN. However, statistically significant differences persisted ($p < 0.0001$), with SA + NZ/FL + CN maintaining the highest residual activity. This indicates that this system provides a more sustained release of bioactive CN, likely due to a balance between controlled diffusion (PCL film) and enhanced matrix hydration (NZ). In contrast, SA/FL + CN consistently showed the lowest activity across all time points ($p < 0.0001$), confirming that the dense and hydrophobic film structure restricts CN diffusion. SA + NZ/FR + CN showed variable behavior, with fluctuations over time, reflecting heterogeneous release and limited effective availability of CN.

The control samples (Fig. 6g) validated the assay with Trolox exhibiting consistently high antioxidant activity, while PBS and DMSO showed negligible values, confirming that the observed activity arises from CN release. Data showed a strong correlation between CN release (Fig. 5) and antioxidant activity (Fig. 6). Systems that promote higher and more accessible CN release (SA/FR + CN and SA + NZ/FL + CN) exhibit greater antioxidant activity, whereas systems with restricted diffusion remain inactive. Notably, SA + NZ/FL + CN demonstrated the most balanced performance, combining sustained CN release with prolonged antioxidant activity, highlighting its potential for applications requiring extended oxidative protection. The DPPH results indicate that antioxidant activity was governed not only by the amount of CN released but also by its effective availability in solution. In contrast, SA/FL + CN

exhibited limited antioxidant activity despite CN release, likely due to restricted diffusion through the hydrophobic PCL film, which reduced the accessibility of CN to the surrounding medium. More broadly, factors such as hydrophobic interactions, phase separation, and matrix retention may further limit CN availability even after release. Additionally, the presence of NZ appears to enhance CN diffusion and stabilization, likely by increasing matrix hydration and reducing hydrophobic aggregation. These findings reinforce the importance of matrix design in controlling not only release kinetics but also the functional bioactivity of the released compounds.

3.4.5.3. Time-kill kinetics. The antibacterial performance of the hydrogels was evaluated using a quantitative shake flask assay under dynamic conditions (Fig. 7) against Gram-positive bacteria (*S. aureus* and *S. epidermidis*) and Gram-negative bacteria (*E. coli* and *P. aeruginosa*). The analysis was conducted over 24 h, with sampling at 1, 2, 4, 6, and 24 h.

For Gram-positive bacteria (Fig. 7a and b), all NZ-containing systems (SA + NZ, SA + NZ/FL, SA + NZ/FR, SA + NZ/FL + CN, and SA + NZ/FR + CN) exhibited complete bacterial reduction ($\approx 100\%$) from the first hour, with this effect being maintained throughout the entire incubation period ($p < 0.0001$). These results confirm the strong bactericidal activity of NZ against Gram-positive bacteria, which is associated with its ability to bind to lipid II and form pores in the cytoplasmic membrane,

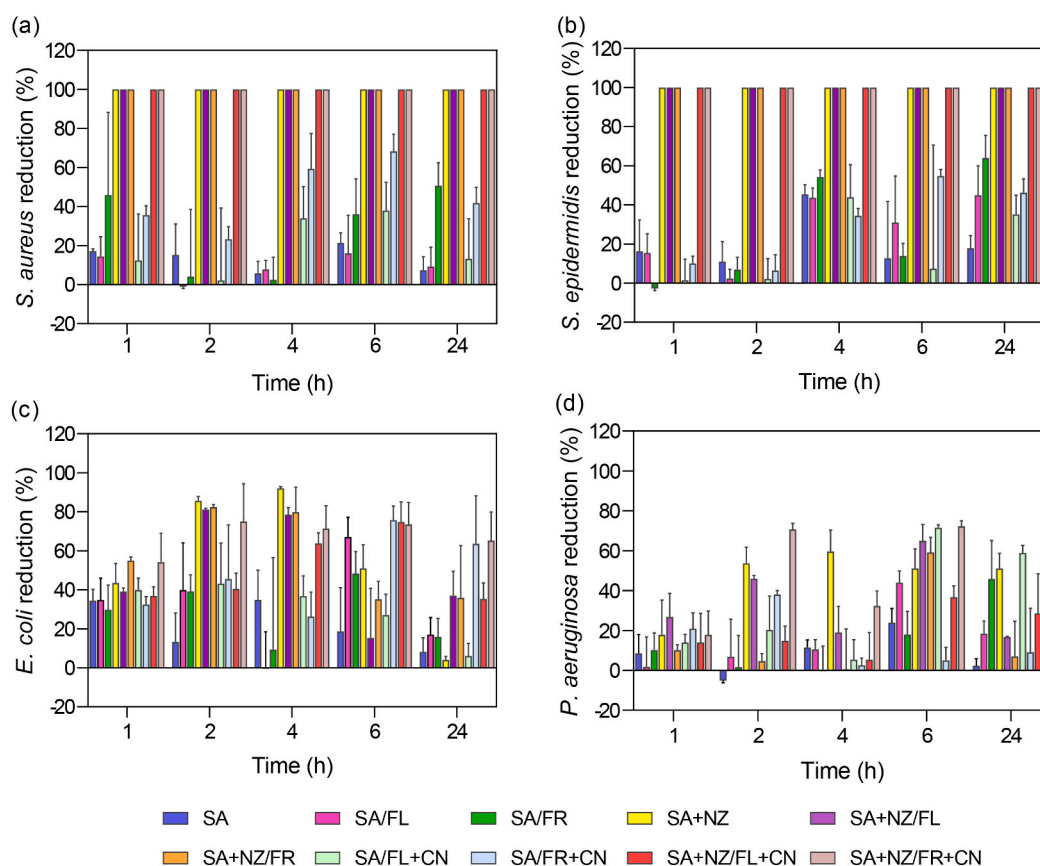


Fig. 7. Reduction in the number (% inhibition) of colonies of bacteria (a) *S. aureus*, (b) *S. epidermidis*, (c) *E. coli* and (d) *P. aeruginosa* after 1, 2, 4, 6, and 24 h of incubation at 37 °C. Data are presented as mean $\pm$ SD ($n = 3$). Statistical significance was assessed using Tukey's multiple comparisons test. Significant differences were observed depending on bacterial strain and incubation time, namely: for Gram-positive bacteria (a,b), $****p < 0.0001$ for almost all comparisons involving NZ-containing systems (SA + NZ, SA + NZ/FL, SA + NZ/FR, SA + NZ/FL + CN and SA + NZ/FR + CN) versus systems without active agents (SA, SA/FL, SA/FR) and CN-only systems (SA/FL + CN and SA/FR + CN); for *E. coli* (c), $****p < 0.0001$ and $***p < 0.001$ were observed for most comparisons between CN/NZ-containing systems (SA + NZ/FL + CN, SA + NZ/FR + CN, SA/FL + CN and SA/FR + CN) and the remaining samples, particularly from 2 h onwards, while early time points showed fewer significant differences; for *P. aeruginosa* (d), significant differences were limited at early time points (mostly non significant), but became evident at longer incubation periods (6 to 24 h), with $**p < 0.01$ to $****p < 0.0001$ observed mainly for comparisons involving CN-containing systems (SA/FL + CN, SA/FR + CN, SA + NZ/FL + CN and SA + NZ/FR + CN) versus the remaining hydrogels. Statistical differences increased with incubation time and were more pronounced in systems containing NZ and/or CN, particularly against Gram-positive bacteria.

leading to rapid cell lysis [32]. The addition of CN (SA + NZ/FL + CN and SA + NZ/FR + CN) did not further increase the reduction level, as complete inhibition was already achieved; however, it likely contributes to reinforcing and stabilizing the antimicrobial effect over longer periods. In contrast, hydrogels without NZ (SA, SA/FL, SA/FR) showed fluctuating and limited antibacterial activity, reflecting the absence of active agents. CN-containing systems without NZ (SA/FL + CN and SA/FR + CN) exhibited moderate activity (≈ 40 –60%), consistent with the known mechanism of CN, which involves disruption of membrane integrity and inhibition of essential enzymatic processes [41–43].

For *E. coli* (Fig. 7c), a more complex and resistant behavior was observed, reflecting the presence of an outer membrane that limits antimicrobial penetration. NZ-containing systems (SA + NZ, SA + NZ/FL, SA + NZ/FR) showed partial reductions (≈ 40 –90%), confirming the limited effectiveness of NZ alone against Gram-negative bacteria. However, when combined with CN (SA + NZ/FL + CN and SA + NZ/FR + CN), a clear enhancement in antibacterial activity was observed, with reductions exceeding 60–70% after 4 to 6 h incubation. This improvement may be attributed to a cooperative mechanism, in which CN disrupts the outer lipid membrane, increasing permeability and facilitating NZ access to intracellular targets. This interpretation is consistent with previous observations in the checkerboard assay (Section 3.1.1) and with literature reports describing membrane permeabilization effects of aromatic aldehydes [64,65]. Hydrogels containing only CN also exhibited antibacterial activity, although less consistent (≈ 10 –70%), while control systems without active agents showed negligible reduction.

For *P. aeruginosa* (Fig. 7d), which is known for its high intrinsic resistance, overall antibacterial activity was lower. NZ-containing systems showed limited effectiveness, with only SA + NZ exhibiting moderate reduction after 24 h. This result is consistent with the absence of synergism observed in the checkerboard assay (Section 3.1.1). On the other hand, CN-containing systems, particularly SA/FL + CN and SA + NZ/FR + CN, achieved reductions above 50% after 24 h, indicating that CN plays a dominant role in antibacterial activity against this strain. Interestingly, the effectiveness of CN-containing systems appears to depend on matrix structure. Systems incorporating fragments generally showed more variable but sometimes enhanced performance, which may be associated with increased surface area and improved release of CN. This observation aligns with the release and antioxidant studies, where matrix morphology was shown to strongly influence the availability of active agents.

Taken across all tested strains, NZ-only systems provided rapid and near-complete bactericidal activity restricted mainly to Gram-positive bacteria, CN-only systems showed more moderate but broader-spectrum activity extending to Gram-negative strains, and the dual NZ/CN systems consistently matched or exceeded the best performance of either single-agent formulation, particularly against the more resistant Gram-negative species.

The results demonstrate that NZ is highly effective against Gram-positive bacteria, while CN plays a crucial role in extending antimicrobial activity to Gram-negative strains. The combination of both agents enables a broader antibacterial spectrum, particularly through mechanisms involving membrane destabilization and enhanced permeability. Additionally, the hydrogel structure itself modulates the efficacy of the system by controlling the release and availability of the active compounds. From an application perspective, these findings highlight the potential of SA-based reinforced hydrogels as multifunctional wound dressings, capable of providing immediate antibacterial action (via NZ) and sustained activity (via CN), while overcoming the intrinsic resistance mechanisms of clinically relevant pathogens. This dual-kinetics profile, with immediate bactericidal action complemented by sustained activity against a broader bacterial spectrum, has rarely been demonstrated within a single fiber-reinforced hydrogel matrix, where most reported systems rely on a single release-controlled agent. The present results indicate that compartmentalizing NZ and CN within

distinct structural elements of the hydrogel enables this complementary kinetic behavior without requiring additional chemical modification of either agent.

3.4.5.4. Biofilm inhibition. Biofilm inhibition was evaluated using two complementary methods: the crystal violet assay and the resazurin assay (Fig. 8), which allow different aspects of the biofilm to be analyzed. The crystal violet assay quantifies the total biomass adhered to the surface, reflecting the extent of biofilm biomass without distinguishing between viable and non-viable cells. In contrast, the resazurin assay evaluates the metabolic activity of the cells embedded within the biofilm, providing insight into bacterial viability. Thus, while the former assesses biofilm formation, the latter reflects the physiological state of the biofilm-associated cells.

In the crystal violet assay (Fig. 8a), a marked inhibition of *S. aureus* biofilms was observed for NZ-containing systems, particularly SA + NZ, SA + NZ/FL, and SA + NZ/FR, reaching values close to 90%. This confirms the strong antibiofilm activity of NZ against Gram-positive bacteria, which is consistent with its mechanism of action involving

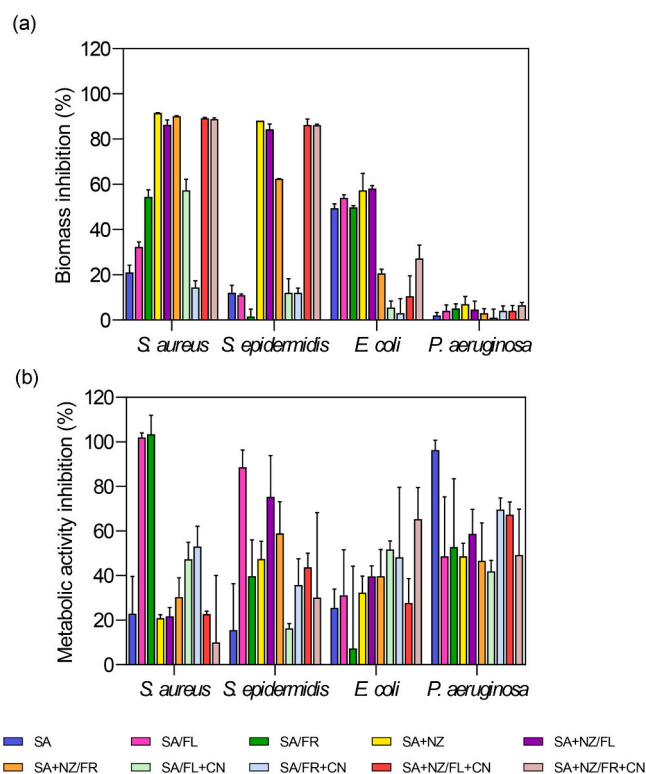


Fig. 8. Inhibition of biofilms formed by each bacterium (individual cultures) after 48 h of incubation in contact with hydrogels: (a) evaluation by the crystal violet assay, reflecting total biofilm biomass, and (b) evaluation by the resazurin assay, indicating metabolic activity (viability) of biofilm-embedded cells. Measurements were performed with samples protected from light. Data are presented as mean $\pm$ SD ($n = 3$). Statistical significance was assessed using Tukey's multiple comparisons test. Significant differences were observed between control hydrogels (SA, SA/FL, SA/FR) and NZ- and/or CN-containing systems. In particular, **** $p < 0.0001$ was observed for comparisons between control formulations and NZ-containing hydrogels (SA + NZ, SA + NZ/FL, SA + NZ/FR), as well as CN-containing systems (SA/FL + CN, SA/FR + CN, SA + NZ/FL + CN, SA + NZ/FR + CN) in *S. aureus* and *S. epidermidis*. For Gram-negative bacteria (*E. coli* and *P. aeruginosa*), significant differences were generally lower, with ** $p < 0.01$ observed mainly for CN-containing systems compared to controls, while * $p < 0.05$ was detected in selected comparisons involving NZ-containing formulations. Differences between NZ-only and CN-only systems were variable and, in some cases, not statistically significant, indicating that the antibiofilm effect depends on both bacterial strain and hydrogel composition.

membrane disruption and pore formation. In contrast, the control hydrogels (SA, SA/FL, and SA/FR) showed limited or negligible inhibition, demonstrating that neither the SA matrix nor the PCL structures contribute to antibiofilm activity in the absence of the active agents. For *E. coli*, inhibition was lower, not exceeding 60% even in the most complex systems. This behavior reflects the intrinsic resistance of Gram-negative bacteria, whose outer membrane acts as an effective barrier to antimicrobial agents, limiting NZ penetration. This trend was even more pronounced for *P. aeruginosa*, where only a few hydrogels exhibited minimal inhibitory activity ($\approx 5\%$), highlighting the well-known tolerance of this species, which is associated with its highly protective biofilm matrix and efficient efflux systems.

The resazurin assay results (Fig. 8b) corroborate these observations, providing additional insight into biofilm viability. Hydrogels without active agents (SA, SA/FL, and SA/FR) showed negligible effects, confirming the lack of intrinsic antibiofilm properties of the polymeric matrix. The incorporation of NZ led to a pronounced reduction in metabolic activity, particularly in Gram-positive bacteria (*S. aureus* and *S. epidermidis*), consistent with its preferential activity against these microorganisms. In Gram-negative bacteria (*E. coli* and *P. aeruginosa*), the reduction in viability was more limited when NZ was used alone, again reflecting the protective role of the outer membrane. However, the incorporation of CN resulted in a more pronounced decrease in viability across all bacterial species. This behavior can be attributed to the hydrophobic nature of CN, which facilitates its interaction with lipid components of bacterial membranes, promoting membrane destabilization and increasing permeability. In addition, CN has been reported to interfere with quorum sensing mechanisms and biofilm maturation, contributing to its antibiofilm effect [46,47].

The combination of NZ and CN (SA + NZ/FL + CN and SA + NZ/FR + CN) demonstrated an overall improvement in antibiofilm performance, particularly against Gram-negative bacteria. This suggests a complementary mechanism, where CN enhances membrane permeability, facilitating NZ access to intracellular targets. However, this effect was not always strictly additive, which may be related to differences in release kinetics, as previously observed, as well as possible competitive interactions within the hydrogel matrix that affect the availability of each agent.

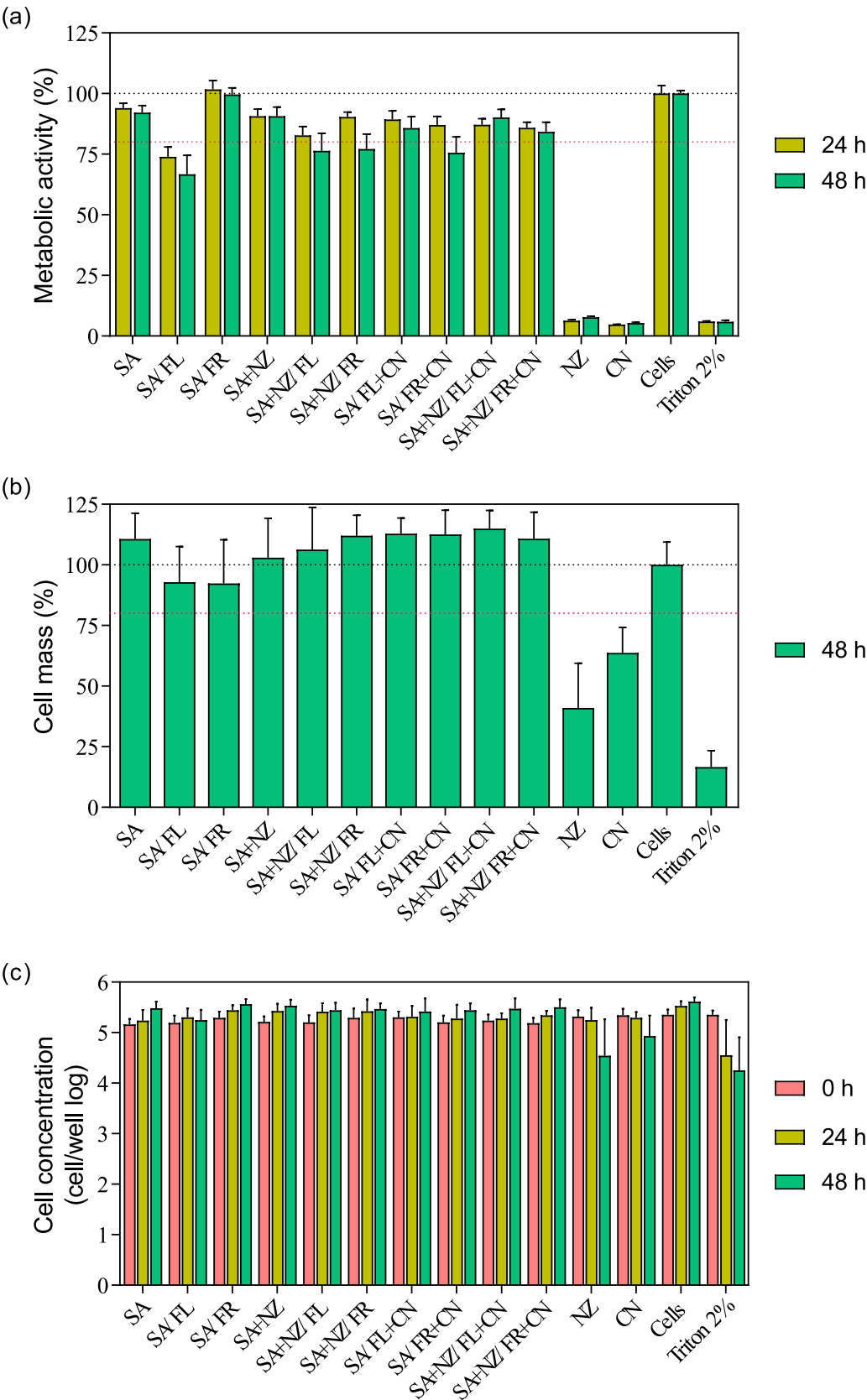
Data demonstrated that hydrogels incorporating NZ and CN exhibit important antibiofilm activity, particularly against Gram-positive bacteria. CN provides broader-spectrum activity, extending effectiveness to Gram-negative species, while the combination of both agents enhances overall performance through complementary mechanisms. These findings highlight the potential of these systems as multifunctional wound dressings capable of preventing biofilm formation and reducing bacterial viability in infected environments. Unlike single-agent SA dressings, which typically show strong antibiofilm activity restricted to Gram-positive strains, the complementary mechanisms of NZ and CN observed here extend meaningful antibiofilm effects toward Gram-negative species as well, a broader spectrum that is directly relevant given the polymicrobial nature of most infected pressure ulcers.

3.4.5.5. Cell response. A wound dressing intended for tissue repair must exhibit high biocompatibility, ensuring that it supports cellular processes involved in regeneration without inducing cytotoxic effects or impairing cell function. In this context, the cytocompatibility of the developed hydrogels was evaluated using a direct contact assay with human keratinocytes (HaCaT), which mimics the interaction between the material and the epidermal layer in vivo [66]. Metabolic activity (Fig. 9a) was assessed at 24 and 48 h as an indicator of cellular metabolic activity and viability, while total cellular protein content (Fig. 9b), associated with protein synthesis by adherent cells, was quantified at 48 h. Since non-adherent cells may still be present at earlier time points, this assay was only performed after prolonged incubation. Additionally, cell proliferation (Fig. 9c) was monitored from the initial contact (0 h)

up to 48 h, providing insight into the ability of the hydrogels to support cell growth over time. According to ISO 10993-5:2009, materials are considered non-cytotoxic when cell viability exceeds 80% [67]. The results demonstrate that the developed hydrogels exhibit good cytocompatibility. Except for the SA/FL sample, all formulations maintained metabolic activity above 83.0% after 24 h (Fig. 9a), indicating that the presence of PCL, NZ, and CN does not induce marked cytotoxic effects. After 48 h, a slight decrease in metabolic activity was observed for SA + NZ/FL, SA + NZ/FR, and SA/FR + CN, with values around 77%. Although slightly below the ISO threshold, this reduction is moderate and should be interpreted in the context of prolonged exposure and increased release of bioactive agents. Importantly, this decrease was not accompanied by a reduction in total protein content, suggesting that cell adhesion was not compromised and that cells remained viable and metabolically active. Indeed, all hydrogels exhibited high cellular protein content ($> 92.3\%$) at 48 h (Fig. 9b), indicating effective cell attachment and proliferation. This observation highlights that metabolic activity and protein production do not necessarily correlate linearly, as cells may maintain proliferation and structural functions even when experiencing mild metabolic stress [68]. This is particularly relevant for biomaterials incorporating bioactive compounds, where transient reductions in metabolic activity may reflect adaptive cellular responses rather than cytotoxicity. The slightly lower metabolic activity observed in NZ-containing hydrogels can be attributed to the cationic nature of antimicrobial peptides. NZ is known to interact with negatively charged phospholipids, which are more abundant in bacterial membranes but are also present, to a lesser extent, in mammalian cells (weaker interactions) [29]. These electrostatic interactions may induce temporary alterations in membrane permeability or ion flux, leading to subtle reductions in mitochondrial activity. However, the absence of significant effects on protein production and proliferation suggest that these interactions are reversible and do not compromise long-term cell viability. Furthermore, NZ is classified as GRAS (Generally Recognized as Safe), reinforcing its suitability for biomedical applications when used within appropriate concentration ranges [30].

It is worth noting that free CN in solution has been reported to induce significant cytotoxicity in skin fibroblasts at concentrations around 100 $\mu\text{g/mL}$ after 24 h of exposure, well below the total CN amount immobilized within the fibrous reinforcement in this study ($2.793 \pm 0.357 \text{ mg/m}$) [69]. However, the high cytocompatibility observed here is consistent with the compartmentalized, sustained release profile of CN from the fiber-reinforced hydrogel (Section 3.4.5.1), which is approximately ten times slower than that of NZ and remains largely below its cytotoxic threshold under the tested conditions. This highlights the relevance of controlled, matrix-mediated delivery in mitigating the cytotoxic potential typically associated with free CN. Similarly, NZ has been reported to be well tolerated by human keratinocytes at low concentrations, consistent with its preferential interaction with bacterial membranes over mammalian cells [70], further supporting the biological safety of the functionalized hydrogels within the concentration ranges used in this study.

The influence of PCL morphology on the cell response was particularly evident in CN-containing systems. In SA/FL + CN hydrogels, the presence of a continuous PCL film provides a more controlled and sustained release of CN, resulting in a more stable microenvironment that supports cellular metabolism. In contrast, SA/FR + CN hydrogels contain PCL fragments with a higher surface area, promoting faster and more localized release of CN. This may lead to higher local concentrations of the compound, explaining the observed decrease in metabolic activity at 48 h. Despite this, the structural characteristics of the fragmented system appear to favor cell adhesion, as reflected by the high protein content. The increased surface roughness and porosity likely enhance cell-material interactions, facilitating nutrient exchange and oxygen diffusion. Interestingly, hydrogels combining NZ and CN (SA + NZ/FL + CN and SA + NZ/FR + CN) showed improved cellular responses, with increased metabolic activity and protein content



(caption on next page)

Fig. 9. Cytocompatibility of hydrogels evaluated by direct contact with human keratinocytes (HaCaT): (a) metabolic activity at 24 and 48 h, (b) total cellular protein content at 48 h, and (c) cell proliferation at 0, 24, and 48 h. Measurements were performed under standard culture conditions. Data are presented as mean $\pm$ SD ($n = 3$). Statistical significance was assessed using Tukey's multiple comparisons test. For metabolic activity (a), at 24 h significant differences were mainly observed for SA/FL, which showed lower values compared to most systems ($****p < 0.0001$), with additional $*p < 0.05$ differences between NZ-containing systems and controls. At 48 h, differences became more pronounced, with $****p < 0.0001$ for comparisons involving SA/FL and NZ-containing hydrogels (SA + NZ/FL and SA + NZ/FR) vs. control systems, $***p < 0.001$ for comparisons involving SA/FR + CN and combined NZ + CN systems, and $*p < 0.05$ differences among remaining formulations. For total protein content (b), $****p < 0.0001$ differences were observed between Triton 2% and all hydrogels, as well as between NZ alone and all formulations; $***p < 0.001$ for CN vs. control hydrogels; and $*p < 0.05$ for comparisons between control hydrogels and reinforced systems, particularly those containing NZ and/or CN. For cell proliferation (c), significant differences were mainly observed at 24 h and 48 h, with $**p < 0.01$ and $*p < 0.05$ for comparisons involving SA + NZ/FR and SA/FL + CN vs. control systems at 24 h, and $****p < 0.0001$ for comparisons between Triton 2% and all samples at 48 h, alongside $***p < 0.001$ and $**p < 0.01$ differences between NZ and CN alone vs. hydrogel systems, and $*p < 0.05$ differences among remaining formulations.

compared to systems containing each agent individually. This suggests a complementary effect between both bioactive compounds. CN, known for its antioxidant properties, may contribute to modulation of oxidative stress and protect cells from potential damage, while NZ, when released in a controlled manner, may act as a mild bioactive stimulus, promoting cellular activity. Additionally, the combined presence of these agents may modulate the local microenvironment by reducing microbial contamination and maintaining conditions favorable for cell growth.

The proliferation data (Fig. 9c) further support these findings, showing a consistent increase in cell number over 48 h for all hydrogels. This trend indicates that the materials not only preserve cell viability but also actively support cell growth. The presence of PCL, whether in film or fragment form, appears to contribute to this effect by providing mechanical support and structural stability, while the hydrated hydrogel matrix ensures a moist environment conducive to cell survival. Together, these features mimic key aspects of the natural extracellular matrix, promoting cell adhesion, spreading, and proliferation. From a broader perspective, the balance between antimicrobial activity and cytocompatibility is critical for wound dressing applications. While strong antimicrobial agents may compromise mammalian cell viability, the results obtained here demonstrate that the incorporation of NZ and CN into SA-based hydrogels achieves an effective compromise. The systems exhibit sufficient antimicrobial potential, as previously demonstrated, while maintaining high levels of cytocompatibility and supporting cellular functions essential for tissue regeneration [48,71]. Although some formulations induced a slight reduction in metabolic activity, this effect was mild and reversible, likely reflecting the controlled release of the incorporated bioactive compounds rather than any cytotoxic response.

The developed hydrogels demonstrate excellent cytocompatibility, with the ability to support keratinocyte adhesion, proliferation, and metabolic activity, while only inducing mild and reversible reductions in metabolic activity associated with the controlled release of bioactive compounds. The combination of structural reinforcement (PCL) and bioactive agents (NZ and CN) results in multifunctional systems capable of promoting a favorable cellular response, highlighting their potential for application in wound healing and tissue regeneration.

4. Conclusions

Pressure ulcers remain a major clinical challenge due to impaired healing and high susceptibility to infection. In this work, multifunctional SA-based hydrogels reinforced with PCL (FL and FR) and loaded with NZ and CN were successfully developed, combining structural support with antimicrobial and bioactive properties.

The hydrogels exhibited homogeneous morphology and effective incorporation of PCL, contributing to improved mechanical performance. FL-reinforced systems maintained higher compressive strength, while FR-reinforced hydrogels showed enhanced elasticity and recovery capacity, which is advantageous under continuous mechanical stress conditions typical of pressure ulcers. Thermal and chemical analyses confirmed the successful incorporation of NZ and CN and their interactions with the SA matrix.

Biological assays demonstrated complementary antimicrobial effects

between NZ and CN. NZ was particularly effective against Gram-positive bacteria, while CN showed broader-spectrum activity, including against Gram-negative strains. Their combination enhanced antibacterial and antibiofilm performance, especially against *Staphylococcus* spp., highlighting a synergistic or additive effect. However, limited efficacy was still observed against *P. aeruginosa*, reflecting its intrinsic resistance.

Cytocompatibility studies confirmed that all hydrogels, except SA/FL, were non-cytotoxic, maintaining metabolic activity above 80% and high cell mass and proliferation levels. Slight reductions in metabolic activity in NZ-containing systems were observed but did not compromise overall viability. The presence of PCL and CN contributed to a favorable microenvironment, supporting cell adhesion, proliferation, and metabolic activity.

Therefore, the developed fiber-reinforced alginate hydrogels successfully combined structural reinforcement with complementary antibacterial and antioxidant functions while exhibiting suitable physicochemical, mechanical, and biological properties for chronic wound applications. Rather than simply incorporating bioactive agents into a hydrogel, the present work demonstrates that reinforcement architecture and compartmentalized bioactive loading can be jointly engineered to obtain multifunctional wound dressings with tailored structural and biological performance. This strategy, combining tunable fiber-based reinforcement with compartmentalized, complementary bioactive delivery, has not been previously reported in alginate-based systems, which to date have typically addressed mechanical reinforcement and dual-agent antimicrobial delivery as separate design goals. It offers a promising platform for next-generation dressings for infected chronic wounds, though the reduced efficacy observed against *P. aeruginosa* indicates that further optimization, for example through agent dose escalation or the inclusion of a Gram-negative-targeted third agent, will be needed before clinical translation.

CRedit authorship contribution statement

Joana Fernandes: Writing – review & editing, Writing – original draft, Data curation. **Micaela Gomes:** Writing – review & editing, Writing – original draft, Data curation. **Ana Rita C. Bastos:** Writing – original draft, Validation, Investigation, Data curation. **Sonia L.C. Pinho:** Writing – review & editing, Methodology, Investigation. **Helena P. Felgueiras:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Helena P. Felgueiras reports financial support was provided by Foundation for Science and Technology. Micaela Gomes reports financial support was provided by Foundation for Science and Technology. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This work was funded by national funds through FCT – Fundação para a Ciência e a Tecnologia, I.P. and Programa de Recuperação e Resiliência (PRR) through NextGenerationEU from the European Union, under the Strategic Projects UID/00264/2025 and UID/PRR/00264/2025 of 2C2T (doi:10.54499/UID/00264/2025 and doi:10.54499/UID/PRR/00264/2025). The authors also thank FCT for the financial support to the Research Centre for Natural Resources, Environment and Society — CERNAS (UIDB/00681/2025, doi:10.54499/UID/00681/2025).

Additionally, authors acknowledge funding received from FCT under the scope of the project 2024.13379.UTA (doi:10.54499/2024.13379.UTA). M.G. acknowledges FCT for PhD funding via scholarship 2023.00804.BDANA, and H.P.F. for an auxiliary researcher contract with reference 2021.02720.CEECIND.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioadv.2026.215171>.

Data availability

No data was used for the research described in the article.

References

- [1] E. Jaul, J. Barron, J.P. Rosenzweig, J. Menczel, An overview of co-morbidities and the development of pressure ulcers among older adults, *BMC Geriatr.* 18 (2018), <https://doi.org/10.1186/s12877-018-0997-7>.
- [2] A. Gefen, Pressure ulcer prevention dressing design and biomechanical efficacy, *J. Wound Care* 29 (2020) S6–15, <https://doi.org/10.12968/jowc.2020.29.Sup12.S6>.
- [3] P.M.G. Sardo, J.P.F. Teixeira, A.M.S.F. Machado, B.F. Oliveira, I.M. Alves, A systematic review of prevalence and incidence of pressure ulcers/injuries in hospital emergency services, *J. Tissue Viability* 32 (2023) 179–187, <https://doi.org/10.1016/j.jtv.2023.02.001>.
- [4] W.V. Padula, B.A. Delarment, The national cost of hospital-acquired pressure injuries in the United States, *Int. Wound J.* 16 (2019) 634–640, <https://doi.org/10.1111/iwj.13071>.
- [5] M. de M.F. Coelho, B.M.A. Avelino, B.A. de Oliveira, M.A. Rios, F.D.A. Gubert, M. C. Martins, et al., Prevalence of biofilm in chronic wounds: systematic review with meta-analysis, *Wounds* 37 (2025) 283–291, <https://doi.org/10.25270/wnds/24124>.
- [6] A.Z. Shen, M. Taha, M. Ghannoum, S.K. Tying, Biofilms and chronic wounds: pathogenesis and treatment options, *J. Clin. Med.* 14 (2025) 7784, <https://doi.org/10.3390/jcm14217784>.
- [7] L. Martinengo, M. Olsson, R. Bajpai, M. Soljak, Z. Upton, A. Schmidtchen, et al., Prevalence of chronic wounds in the general population: systematic review and meta-analysis of observational studies, *Ann. Epidemiol.* 29 (2019) 8–15, <https://doi.org/10.1016/j.annepidem.2018.10.005>.
- [8] A. Miron, C. Giurcaneanu, M.M. Mihai, C. Beiu, V.M. Voiculescu, M.N. Popescu, et al., Antimicrobial biomaterials for chronic wound care, *Pharmaceutics* 15 (2023), <https://doi.org/10.3390/pharmaceutics15061606>.
- [9] D. Solanki, P. Vinchi, M.M. Patel, Design considerations, formulation approaches, and strategic advances of hydrogel dressings for chronic wound management, *ACS Omega* 8 (2023) 8172–8189, <https://doi.org/10.1021/acsomega.2c06806>.
- [10] Y. Liang, J. He, B. Guo, Functional hydrogels as wound dressing to enhance wound healing, *ACS Nano* 15 (2021) 12687–12722, <https://doi.org/10.1021/acsnano.1c04206>.
- [11] Y. Hu, L. Yu, Q. Dai, X. Hu, Y. Shen, Y. Shen, Multifunctional antibacterial hydrogels for chronic wound management, *Biomater. Sci.* 12 (2024) 2460–2479, <https://doi.org/10.1039/d4bm00155a>.
- [12] V. Gounden, M. Singh, Hydrogels and wound healing: current and future prospects, *Gels* 10 (2024) 43, <https://doi.org/10.3390/gels10010043>.
- [13] B.A. Aderibigbe, B. Buyana, Alginate in wound dressings, *Pharmaceutics* 10 (2018) 42, <https://doi.org/10.3390/pharmaceutics10020042>.
- [14] Z. Sarwar, N. Aleem, F. Azam, F. Ahmad, B. Mushtaq, S. Ahmad, et al., Sustainable alginate hydrogel fibers reinforced with cotton and bamboo for high wound exudates, *Ind. Crop. Prod.* 237 (2025) 122185, <https://doi.org/10.1016/j.indcrop.2025.122185>.
- [15] A.L. Alfaluji, Q.S. Kadhim, A.A.A. Mahdi, Electrospun poly(ϵ -caprolactone)/silver nanoparticle nanofibrous scaffolds with antibacterial activity for wound-dressing applications, *RSC Adv.* 15 (2025) 37899–37907, <https://doi.org/10.1039/d5ra06746d>.
- [16] G.K. Sharma, N.R. James, G.K. Sharma, N.R. James, Electrospinning: the technique and applications, in: *Recent Developments in Nanofibers Research*, 2022, <https://doi.org/10.5772/intechopen.105804>.
- [17] M. Gizaw, J. Thompson, A. Faglie, S.Y. Lee, P. Neuenschwander, S.F. Chou, Electrospun fibers as a dressing material for drug and biological agent delivery in wound healing applications, *Bioengineering* (Basel, Switzerland) 5 (2018) 9, <https://doi.org/10.3390/bioengineering5010009>.
- [18] S. Nagarajan, C. Pochat-Bohatier, S. Balme, P. Miele, S.N. Kalkura, M. Bechelany, Electrospun fibers in regenerative tissue engineering and drug delivery, *Pure Appl. Chem.* 89 (2017) 1799–1808, <https://doi.org/10.1515/pac-2017-0511>.
- [19] P.C. Fuioga, D.M. Rata, T. Riaz, G. Rivero, G.A. Abraham, L.I. Atanase, Composite hydrogels with embedded electrospun fibers as drug delivery systems, *Gels* 11 (2025) 826, <https://doi.org/10.3390/gels1100826>.
- [20] A.D. Tsareva, V.S. Shtol, D.V. Klinov, D.A. Ivanov, Electrospinning for biomedical applications: an overview of material fabrication techniques, *Surfaces* 8 (2025) 7, <https://doi.org/10.3390/surfaces8010007>.
- [21] K. Ye, H. Kuang, Z. You, Y. Morsi, X. Mo, Electrospun nanofibers for tissue engineering with drug loading and release, *Pharmaceutics* 11 (2019) 182, <https://doi.org/10.3390/pharmaceutics11040182>.
- [22] F.M. Chen, X. Liu, Advancing biomaterials of human origin for tissue engineering, *Prog. Polym. Sci.* 53 (2016) 86–168, <https://doi.org/10.1016/j.progpolymsci.2015.02.004>.
- [23] E. Marinho, B.M. Silva, C.S. Miranda, S.L.C. Pinho, H.P. Felgueiras, Polycaprolactone/sodium alginate coaxial wet-spun fibers modified with carbon nanofibers and ceftazidime for improved clotting and infection control in wounds, *Biomater. Sci.* 13 (2025) 2047–2065, <https://doi.org/10.1039/d4bm01667j>.
- [24] A.R.M. Ribeiro, M.O. Teixeira, L. Ribeiro, T.D. Tavares, C.S. Miranda, A.F. Costa, et al., Sodium alginate-based multifunctional sandwich-like system for treating wound infections, *Biomater. Adv.* 162 (2024), <https://doi.org/10.1016/j.bioadv.2024.213931>.
- [25] S.M. Saeed, H. Mirzadeh, M. Zandi, J. Barzin, Designing and fabrication of curcumin loaded PCL/PVA multi-layer nanofibrous electrospun structures as active wound dressing, *Prog. Biomater.* 6 (1) (2017) 39–48, <https://doi.org/10.1007/s40204-017-0062-1>.
- [26] G. Zhang, T. Li, J. Liu, X. Wu, H. Yi, Cinnamaldehyde-contained polymers and their biomedical applications, *Polymers* (Basel) 15 (2023) 1517, <https://doi.org/10.3390/polym15061517>.
- [27] S.B. Adnan, M. Maarof, M.B. Fauzi, N.I. Md Fadilah, Antimicrobial peptides in wound healing and skin regeneration: dual roles in immunity and microbial defense, *Int. J. Mol. Sci.* 26 (2025) 5920, <https://doi.org/10.3390/ijms26135920>.
- [28] H.P. Felgueiras, M.T.P. Amorim, Pioneering wound care solutions: triaxial wet-spun fibers with bioactive agents for chronic wounds, part II (controlled release and biological activity of the active agents), *Mater. Adv.* 6 (2025) 2029–2047, <https://doi.org/10.1016/j.colsurf.2017.05.001>.
- [29] T.D. Tavares, A.R.M. Ribeiro, C. Silva, J.C. Antunes, H.P. Felgueiras, Combinatory effect of nisin antimicrobial peptide with bioactive molecules: a review, *J. Drug Deliv. Sci. Technol.* 91 (2024), <https://doi.org/10.1016/j.jddst.2023.105246>.
- [30] T.D. Tavares, S.L.C. Pinho, E.J. Delgado-Pujol, B. Begines, A. Alcudia, C. Silva, et al., Layer-by-layer electrospun PCL/PVA mats loaded with nisin Z for enhanced diabetic foot ulcer treatment, *Int. J. Pharm.* 685 (2025), <https://doi.org/10.1016/j.ijpharm.2025.126208>.
- [31] A. Joy, N. Jerome, S.U. Ramakrishnan, S. Jeyaraman, D. Vijayakumar, C. Vidjeyamannane, et al., Nano delivery platforms for nisin, a potent cyclic peptide: a comprehensive review of biological applications, *Polym. Bull.* 82 (2025) 4505–4530, <https://doi.org/10.1007/s00289-025-05716-4>.
- [32] N.C. Homem, T.D. Tavares, C.S. Miranda, J.C. Antunes, M.T.P. Amorim, H. P. Felgueiras, Functionalization of crosslinked sodium alginate/gelatin wet-spun porous fibers with nisin Z for the inhibition of *Staphylococcus aureus*-induced infections, *Int. J. Mol. Sci.* 22 (2021) 1930, <https://doi.org/10.3390/ijms22041930>.
- [33] K.A. Qureshi, S.A.A. Mohammed, O. Khan, H.M. Ali, M.Z. El-Readi, H. A. Mohammed, Cinnamaldehyde-based self-nanoemulsion (CA-SNEDDS) accelerates wound healing and exerts antimicrobial, antioxidant, and anti-inflammatory effects in rats' skin burn model, *Molecules* 27 (2022) 5225, <https://doi.org/10.3390/molecules27165225>.
- [34] M. Osanloo, F. Noori, A. Tavassoli, M. reza Ataollahi, A. Davoodi, M. Seifalah-Zade, et al., Effect of PCL Nanofiber Mats Coated With Chitosan Microcapsules Containing Cinnamon Essential Oil for Wound Healing, 2022, <https://doi.org/10.21203/rs.3.rs-2229385/v1>.
- [35] B. Zhou, C. Zhang, S. Dai, J. Zhao, H. Li, Y. Peng, et al., Injectable cinnamaldehyde-loaded ZIF-8/gallic acid-grafted gelatin hydrogel for enhanced angiogenesis and skin regeneration in diabetic wound healing, *Front. Bioeng. Biotechnol.* 13 (2025), <https://doi.org/10.3389/fbioe.2025.1660821>.
- [36] J.G.F. Garrido, N. Andreo Filho, F. Perrechil, M.A. de Moraes, Advanced biomaterial for dual-drug release: a hydrogel-microparticle approach, *Biopolymers* 116 (2025) e70049, <https://doi.org/10.1002/BIP.70049;REQUESTEDJOURNAL:JOURNAL:10970282;CTYPE:STRING:JOURNAL>.
- [37] D. An, Z. Wang, Y. Ning, Y. Yue, H. Xuan, Y. Hu, et al., One-step physical and chemical dual-reinforcement with hydrophobic drug delivery in gelatin hydrogels for antibacterial wound healing, *ACS Omega* 9 (2024) 34413–34427, <https://doi.org/10.1021/ACSOMEGA.4C01963>.
- [38] D. Michiu, M.I. Socaciu, M. Fogarasi, A.M. Jimborean, F. Ranga, V. Mureșan, et al., Implementation of an analytical method for spectrophotometric evaluation of total phenolic content in essential oils, *Molecules* 27 (2022), <https://doi.org/10.3390/molecules27041345>.

- [39] W. Brand-Williams, M.E. Cuvelier, C. Berset, Use of a free radical method to evaluate antioxidant activity, *LWT Food Sci. Technol.* 28 (1995) 25–30, [https://doi.org/10.1016/S0023-6438\(95\)80008-5](https://doi.org/10.1016/S0023-6438(95)80008-5).
- [40] A.R.M. Ribeiro, C.S. Miranda, A.F.G. Silva, F.D.P. Mendes, B.M. Silva, B.A. S. Oliveira, et al., Inhibition of enzyme and bacteria activities in diabetic ulcer-like scenarios via WAAPV-loaded electrospun fibers, *Pharmaceutics* 16 (2024), <https://doi.org/10.3390/pharmaceutics16070911>.
- [41] S. Shen, T. Zhang, Y. Yuan, S. Lin, J. Xu, H. Ye, Effects of cinnamaldehyde on *Escherichia coli* and *Staphylococcus aureus* membrane, *Food Control* 47 (2015) 196–202, <https://doi.org/10.1016/j.foodcont.2014.07.003>.
- [42] M. Albano, B.P. Crulhas, F.C.B. Alves, A.F.M. Pereira, B.F.M.T. Andrade, L. N. Barbosa, et al., Antibacterial and anti-biofilm activities of cinnamaldehyde against *S. epidermidis*, *Microb. Pathog.* 126 (2019) 231–238, <https://doi.org/10.1016/j.micpath.2018.11.009>.
- [43] T.A.F. Ferro, J.M.M. Araújo, B.L. dos S. Pinto, J.S. dos Santos, E.B. Souza, B.L.R. da Silva, et al., Cinnamaldehyde inhibits *Staphylococcus aureus* virulence factors and protects against infection in a *Galleria mellonella* model, *Front. Microbiol.* 7 (2016), <https://doi.org/10.3389/fmicb.2016.02052>.
- [44] D.F. Firmino, T.T.A. Cavalcante, G.A. Gomes, N.C.S. Firmino, L.D. Rosa, M.G. De Carvalho, et al., Antibacterial and antibiofilm activities of *Cinnamomum* sp. essential oil and cinnamaldehyde: antimicrobial activities, *Sci. World J.* 2018 (2018), <https://doi.org/10.1155/2018/7405736>.
- [45] N.G. Vasconcelos, J. Croda, S. Simonatto, Antibacterial mechanisms of cinnamon and its constituents: a review, *Microb. Pathog.* 120 (2018) 198–203, <https://doi.org/10.1016/j.micpath.2018.04.036>.
- [46] R. Karimirad, B.S. Inbaraj, B.H. Chen, Recent advances on the analysis and biological functions of cinnamaldehyde and its derivatives, *Antioxidants* 14 (2025) 765, <https://doi.org/10.3390/antiox14070765>.
- [47] V. Suryanti, F.R. Wibowo, S. Khotijah, N. Andalucki, Antioxidant activities of cinnamaldehyde derivatives, in: *IOP Conf. Ser. Mater. Sci. Eng.* vol. 333, Institute of Physics Publishing, 2018, <https://doi.org/10.1088/1757-899X/333/1/012077>.
- [48] T.D. Tavares, S.L.C. Pinho, E.J. Delgado-Pujol, B. Begines, A. Alcudia, C. Silva, et al., Layer-by-layer electrospun PCL/PVA mats loaded with nisin Z for enhanced diabetic foot ulcer treatment, *Int. J. Pharm.* 685 (2025), <https://doi.org/10.1016/j.ijpharm.2025.126208>.
- [49] J. Xie, K.M. Schaich, Re-evaluation of the 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) assay for antioxidant activity, *J. Agric. Food Chem.* 62 (2014) 4251–4260, <https://doi.org/10.1021/jf500180u>.
- [50] S.Y. Kim, K. Muthuramalingam, H.J. Lee, Effects of fragmented polycaprolactone electrospun nanofiber in a hyaluronic acid hydrogel on fibroblasts, *Tissue Cell* 91 (2024), <https://doi.org/10.1016/j.tice.2024.102582>.
- [51] S.R. Derkach, N.G. Voron'ko, N.I. Sokolan, D.S. Kolotova, Y.A. Kuchina, Interactions between gelatin and sodium alginate: UV and FTIR studies, *J. Dispers. Sci. Technol.* 41 (2020) 690–698, <https://doi.org/10.1080/01932691.2019.1611437>.
- [52] A.R.M. Ribeiro, M.O. Teixeira, L. Ribeiro, T.D. Tavares, C.S. Miranda, A.F. Costa, et al., Sodium alginate-based multifunctional sandwich-like system for treating wound infections, *Biomater. Adv.* 162 (2024), <https://doi.org/10.1016/j.bioadv.2024.213931>.
- [53] F.A. Al-Bayati, M.J. Mohammed, Isolation, identification, and purification of cinnamaldehyde from *Cinnamomum zeylanicum* bark oil. An antibacterial study, *Pharm. Biol.* 47 (2009) 61–66, <https://doi.org/10.1080/13880200802430607>.
- [54] A.M. Piccolotto, A.M. Ariati, J.M.A.G. Dos Santos, L.M.S.D.S. Franciscato, M.R. da Silva, P.R.S. Bittencourt, et al., Comparative study of the thermal stability of cinnamon and oregano essential oils from cinnamon and oregano after heat treatment, *Orbital Electron. J. Chem.* 17 (2025) 20–34, <https://doi.org/10.17807/orbital.v17i1.21688>.
- [55] E.M. Ahmed, Hydrogel: preparation, characterization, and applications: a review, *J. Adv. Res.* 6 (2015) 105–121, <https://doi.org/10.1016/j.jare.2013.07.006>.
- [56] C.G. Flores-Hernández, M. de los A. Cornejo-Villegas, A. Moreno-Martell, A. Del Real, Synthesis of a biodegradable polymer of poly (sodium alginate/ethyl acrylate), *Polymers* 13 (2021) 504, <https://doi.org/10.3390/polym13040504>.
- [57] C.M.B. Pinilla, H.A. Isaia, A. Brandelli, Development and characterization of innovative polymeric oil-core nanocarriers for nisin delivery, *Lett. Appl. Nanobiosci.* 12 (2023), <https://doi.org/10.33263/LIANBS121.018>.
- [58] A.A. Doyle, J.C. Stephens, A review of cinnamaldehyde and its derivatives as antibacterial agents, *Fitoterapia* 139 (2019), <https://doi.org/10.1016/j.fitote.2019.104405>.
- [59] S. Mokhtarzadeh, F. Hakimpour, R. Sarvari, S. Agbolaghi, Y. Mansourpanah, Nanocomposite membranes based on sodium alginate/poly(ϵ -caprolactone)/graphene oxide for methanol, ethanol and isopropanol dehydration via pervaporation, *Polym. Bull.* 77 (2020) 3367–3387, <https://doi.org/10.1007/s00289-019-02921-w>.
- [60] J. Mu, S. Li, Z. Zhang, Z. Li, S. Li, Z. Li, et al., Phase-separated hydrogels for advanced biomedical engineering: from material design to applications, *Mater. Today Bio* 38 (2026) 103096, <https://doi.org/10.1016/j.mtbi.2026.103096>.
- [61] Q. Sun, Y.N. Chen, Y.Z. Liu, The effects of external interfaces on hydrophobic interactions I: smooth surface, *Molecules* 29 (2024), <https://doi.org/10.3390/molecules29133128>.
- [62] P. Lu, D. Ruan, M. Huang, M. Tian, K. Zhu, Z. Gan, et al., Harnessing the potential of hydrogels for advanced therapeutic applications: current achievements and future directions, *Signal Transduct. Target. Ther.* 9 (2024), <https://doi.org/10.1038/s41392-024-01852-x>.
- [63] H. Chijcheapaza-Flores, N. Tabary, F. Chai, M. Maton, J.N. Staelens, F. Cazaux, et al., Injectable chitosan-based hydrogels for trans-cinnamaldehyde delivery in the treatment of diabetic foot ulcer infections, *Gels* 9 (2023), <https://doi.org/10.3390/gels9030262>.
- [64] T.J. Montville, Y. Chen, Mechanistic action of pediocin and nisin: recent progress and unresolved questions, *Appl. Microbiol. Biotechnol.* 50 (1998) 511–519, <https://doi.org/10.1007/S002530051328/METRICS>.
- [65] S. Bennett, L. Ben Said, P. Lacasse, F. Malouin, I. Fliss, Susceptibility to nisin, bactofencin, pediocin and reuterin of multidrug resistant *Staphylococcus aureus*, *Streptococcus dysgalactiae* and *Streptococcus uberis* causing bovine mastitis, *Antibiotics* 10 (2021) 1418, <https://doi.org/10.3390/ANTIBIOTICS10111418>.
- [66] S. Tahr, M. Maarof, S. Masri, R. Che Man, H. Masmoudi, M.B. Fauzi, Human epidermal keratinocytes and human dermal fibroblasts interactions seeded on gelatin hydrogel for future application in skin in vitro 3-dimensional model, *Front. Bioeng. Biotechnol.* 11 (2023) 1200618, <https://doi.org/10.3389/FBIOE.2023.1200618/TEXT>.
- [67] M.N.P. Vidal, J.M. Granjeiro, M.N.P. Vidal, J.M. Granjeiro, Cytotoxicity tests for evaluating medical devices: an alert for the development of biotechnology health products, *J. Biomed. Sci. Eng.* 10 (2017) 431–443, <https://doi.org/10.4236/JBSE.2017.109033>.
- [68] K.E. Wellen, C.B. Thompson, Cellular metabolic stress: considering how cells respond to nutrient excess, *Mol. Cell* 40 (2010) 323, <https://doi.org/10.1016/J.MOLCEL.2010.10.004>.
- [69] S. Ranjithkar, D. Zhang, F. Sun, S. Salman, W. He, K. Venkitanarayanan, et al., Cytotoxic effects on cancerous and non-cancerous cells of trans-cinnamaldehyde, carvacrol, and eugenol, *Sci. Rep.* 11 (1) (2021) 16281, <https://doi.org/10.1038/s41598-021-95394-9>.
- [70] I. Serrano, B. Alinhho, E. Cunha, L. Tavares, A. Trindade, M. Oliveira, Bacteriostatic and antibiofilm efficacy of a nisin Z solution against co-cultures of *Staphylococcus aureus* and *Pseudomonas aeruginosa* from diabetic foot infections, *Life* 13 (2023) 504, <https://doi.org/10.3390/LIFE13020504>.
- [71] H.P. Felgueiras, M.T.P. Amorim, Pioneering wound care solutions: triaxial wet-spun fibers with bioactive agents for chronic wounds, part II (controlled release and biological activity of the active agents), *Mater. Adv.* 6 (2025) 2029–2047, <https://doi.org/10.1016/j.colsurf.2017.05.001>.