

Angiología

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biomecánica de andamios
nanocompuestos de
quitosano/nanocelulosa
reticulados con genipina para el
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Diseño y caracterización biomecánica de andamios nanocompuestos de quitosano/nanocelulosa reticulados con genipina para el tratamiento de úlceras del pie diabético

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ABSTRACT

Introduction and objective: the objective of this study was to develop and characterize novel biomimetic nanocomposite scaffolds based on chitosan (CS) and cellulose nanocrystals (CNCs), cross-linked with genipin (GP), specifically designed for the management of diabetic foot ulcers (DFUs). The research focused on tuning the biomechanical properties and exudate absorption capacity to match the requirements of the chronic wound microenvironment.

Materials and methods: dressings were fabricated via a chemical cross-linking method followed by a two-step freezing and lyophilization process. CNCs were incorporated at varying concentrations (10 % and 50 % w/w) to reinforce the CS/GP matrix. The formulations were characterized through X-ray diffraction (XRD) for crystallinity and Scanning Electron Microscopy (SEM) for morphological analysis. Performance was evaluated via swelling assays in PBS, mechanical compression testing (compressive modulus), and in vitro cytocompatibility using 3T3 fibroblasts (MTT assay). Antibacterial properties on five potential pathogenic bacterial species were determined by plate count agar method.

Results: all scaffolds exhibited a highly interconnected honeycomb-like porous structure. The CS/10CNC group showed the highest swelling ratio (~3300 %), while the CS/50CNC group provided superior structural stability with a stable absorption of ~2000 %. Mechanical testing revealed that 50 % CNC incorporation significantly

reinforced the matrix, increasing the compressive modulus (E_c) to 0.45 ± 0.05 MPa (a 5.6-fold increase over the control), achieving a "mechanical match" with human dermal tissue. XRD confirmed the successful integration of crystalline Cellulose I domains. Finally, all formulations supported high cell viability ($> 90\%$), confirming the non-toxic nature of the genipin-crosslinked system. Furthermore, all formulations showed bactericidal properties.

Conclusions: the synthesized CS/GP/CNC nanocomposites represent a promising advanced dressing for DFU treatment. The ability to precisely tune mechanical stiffness and fluid management through CNC concentration, combined with excellent biocompatibility and structural integrity, makes these scaffolds ideal candidates for promoting healing in chronic, highly exudative diabetic wounds.

Keywords: Diabetic foot ulcer. Bio-inspired scaffold. Mechanical matching. Exudate management. Antimicrobial activity. Biocompatibility.

RESUMEN

Introducción y objetivo: el objetivo de este estudio fue desarrollar y caracterizar nuevos andamiajes (*scaffolds*) nanocompuestos biomiméticos basados en quitosano (CS) y nanocristales de celulosa (CNC), reticulados con genipina (GP) y diseñados específicamente para el manejo de las úlceras de pie diabético (UPD). La investigación se centró en ajustar las propiedades biomecánicas y la capacidad de absorción de exudado para satisfacer los requisitos del microentorno de las heridas crónicas.

Materiales y métodos: los apósitos se fabricaron mediante un método de reticulación química seguido de un proceso de congelación y de liofilización en dos etapas. Se incorporaron CNC en diferentes concentraciones (10 % y 50 % p/p) para reforzar la matriz de CS/GP. Las formulaciones se caracterizaron mediante difracción de rayos X

(DRX) para determinar la cristalinidad y la microscopía electrónica de barrido (SEM) para el análisis morfológico. El rendimiento se evaluó mediante ensayos de hinchamiento en PBS, pruebas de compresión mecánica (módulo de compresión) y citocompatibilidad *in vitro* utilizando fibroblastos 3T3 (ensayo MTT). Las propiedades antibacterianas frente a cinco especies bacterianas potencialmente patógenas se determinaron mediante el método de recuento en placa en agar.

Resultados: todos los andamiajes exhibieron una estructura porosa de tipo panal altamente interconectada. El grupo CS/10CNC mostró el mayor índice de hinchamiento (~3300 %), mientras que el grupo CS/50CNC proporcionó una estabilidad estructural superior, con una absorción estable de ~2000 %. Las pruebas mecánicas revelaron que la incorporación del 50 % de CNC reforzó significativamente la matriz, aumentando el módulo de compresión (E_c) a $0,45 \pm 0,05$ MPa (un incremento de 5,6 veces respecto al control), logrando un “ajuste mecánico” con el tejido dérmico humano. La DRX confirmó la integración exitosa de los dominios cristalinos de celulosa de tipo I. Finalmente, todas las formulaciones permitieron una alta viabilidad celular (> 90 %), lo que confirma la naturaleza no tóxica del sistema reticulado con genipina. Además, todas las formulaciones mostraron propiedades bactericidas.

Conclusiones: los nanocompuestos de CS/GP/CNC sintetizados representan un apósito avanzado prometedor para el tratamiento de las UPD. La capacidad de ajustar con precisión la rigidez mecánica y el manejo de fluidos mediante la concentración de CNC, combinada con una excelente biocompatibilidad e integridad estructural, convierte a estos andamiajes en candidatos ideales para promover la cicatrización en heridas diabéticas crónicas y altamente exudativas.

Palabras clave: Úlcera de pie diabético. Andamiaje bioinspirado. Ajuste mecánico. Gestión de exudados. Actividad antimicrobiana. Biocompatibilidad.

INTRODUCTION

Chronic diabetic foot ulcers (DFUs) represent a severe clinical challenge characterized by delayed healing, persistent bacterial colonization/infection, and a high risk of lower-limb amputation (1,2). Effective management of DFUs requires advanced wound dressings capable of absorbing excess fluid, preventing infection, and providing a biomimetic microenvironment for cellular repair. Among naturally derived polymers, chitosan (CS) has been extensively investigated for wound care due to its inherent biocompatibility, hemostatic properties, antimicrobial potential, and structural similarity to the glycosaminoglycans of the extracellular matrix (ECM) (3). Furthermore, its ability to be processed into highly porous, three-dimensional architectures makes it an ideal candidate for facilitating gas exchange and nutrient diffusion in ischemic diabetic tissues.

Despite its biological advantages, the clinical application of pristine chitosan hydrogels is often limited by their poor mechanical stability and rapid dissolution in highly exudative environments. To address this, chemical cross-linking is required to maintain the structural integrity of the scaffold. Genipin (GP), a naturally occurring cross-linking agent derived from *Gardenia jasminoides* Ellis, has emerged as a superior alternative to traditional synthetic cross-linkers (e.g., glutaraldehyde) due to its extremely low cytotoxicity and its ability to form stable covalent networks with the primary amino groups of chitosan (4). Genipin-cross-linked chitosan hydrogels exhibit enhanced physiological stability and biocompatibility, which are essential for prolonged contact with vulnerable ulcerated tissues.

However, while GP cross-linking effectively stabilizes the chitosan matrix, it often lacks the mechanical robustness required to fully withstand the dynamic stresses of patient ambulation. Achieving a precise biomechanical match with human skin while preserving high porosity remains a challenge for pristine hydrogels. To bridge this

gap, the incorporation of cellulose nanocrystals (CNCs) has emerged as a promising strategy to provide structural reinforcement without compromising the scaffold's biological performance.

While GP cross-linking stabilizes the chitosan matrix, DFUs are subjected to continuous mechanical stress during patient ambulation, requiring dressings that match the biomechanical properties of native skin. Clinical performance heavily depends on aligning the compressive modulus of the 3D scaffold with the surrounding soft tissue mechanics (typically 0.1–2.0 MPa) to prevent secondary tissue damage and localized pressure necrosis (5). In this context, the incorporation of cellulose nanocrystals (CNCs) has attracted significant attention. CNCs possess remarkable mechanical stiffness, a highly crystalline structure, and abundant surface hydroxyl groups that can form extensive hydrogen-bonding networks within the chitosan matrix (6). Beyond acting as a mechanical reinforcing agent, the highly hydrophilic nature of CNCs allows for the precise tuning of the scaffold's swelling ratio and exudate absorption capacity without compromising its structural architecture.

Furthermore, although several chitosan-based dressings are currently commercially available, achieving an optimal balance between massive exudate absorption capacity and mechanical stability under physiological loading remains a significant challenge. Most existing options fail to maintain their structural integrity during patient ambulation, often leading to premature scaffold collapse or maceration. This study addresses this limitation by developing a nanocomposite system specifically engineered to withstand mechanical stress without compromising its fluid management properties.

Therefore, the objective of this study was to develop and systematically evaluate novel antibacterial, biomimetic CS/GP/CNC nanocomposite sponges designed specifically for DFU management (7). We hypothesized that varying the concentration of CNCs would allow for the precise tuning of both the mechanical stiffness and the

fluid absorption profile of the scaffolds. To test this, we performed a comprehensive structural characterization (XRD, SEM), evaluated their swelling behavior, and conducted rigorous mechanical testing under compression. Finally, the *in vitro* cytocompatibility using 3T3 fibroblasts was assessed to confirm the potential of these nanocomposites as advanced and mechanically functional wound dressings.

MATERIALS AND METHODS

Medium molecular weight chitosan (CS, CAS: 9012-76-4, product ref. 448877) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Genipin (purity ≥ 98 %) was purchased from Apollo Scientific (USA) (product ref. BUP00080). Cellulose nanocrystals (CNCs, dry powder, product ref. NG01NC0101) were supplied by Nanografi Nano Technology (Ankara, TUR). All other chemical reagents and solvents used in the study were of analytical grade and used as received without further purification.

PREPARATION OF NANOCOMPOSITE SCAFFOLDS

Cross-linked chitosan-based scaffolds were fabricated via a chemical cross-linking method utilizing genipin (GP) as the cross-linking agent (Fig. 1). Briefly, medium molecular weight chitosan (CS) was dissolved in a 1 % (v/v) lactic acid solution to obtain a 2 % (w/v) CS stock at pH 4.5. Separately, a 1 % (w/v) genipin solution was prepared in ultrapure water. To synthesize the CS/GP matrices, the GP solution was added dropwise to 4 mL of the CS solution under constant magnetic stirring at room temperature for 30 min. A final concentration of 1 % (w/w) GP relative to the CS dry mass content was maintained throughout the study. To enhance the liquid absorption capacity and biological response of the scaffolds, cellulose nanocrystals (CNCs) were incorporated such that their content represents 10 % and 50 % (w/w) relative to the total dry polymer mass (CS + CNC), yielding CS:CNC dry weight ratios of 90:10 and

50:50, respectively (Table 1). A 2 wt % CNC aqueous suspension was previously sonicated in an ultrasonic bath to ensure complete and homogeneous dispersion before being blended with the CS/GP mixture. During the entire preparation process, the pH of the reaction mixture was meticulously monitored and maintained within the range of 6.4 – 6.5, which is considered optimal for promoting physiological wound healing.

Table 1. Detailed composition and nomenclature of the prepared CS/GP/CNC nanocomposite scaffolds

Formulation code	Chitosan (wt %)	Genipin (wt %)*	CNC (wt %)**	CS:CNC ratio
CS/GP	100	1	0	100:0
CS/10CNC	90	1	10	90:10
CS/50CNC	50	1	50	50:50

*Relative to chitosan weight; **Relative to total polymer weight (CS + CNC).

The resulting mixtures were subjected to a two-step freezing and lyophilization process to generate the porous architecture. First, samples were frozen at -20 °C for 24 h. Subsequently, they were lyophilized at -80 °C under a controlled pressure (vacuum) for 48 h (Fig. 1). This procedure yielded flexible, highly porous, sponge-like patches capable of conforming to irregular wound topographies, specifically designed for advanced diabetic foot ulcer (DFU) applications.

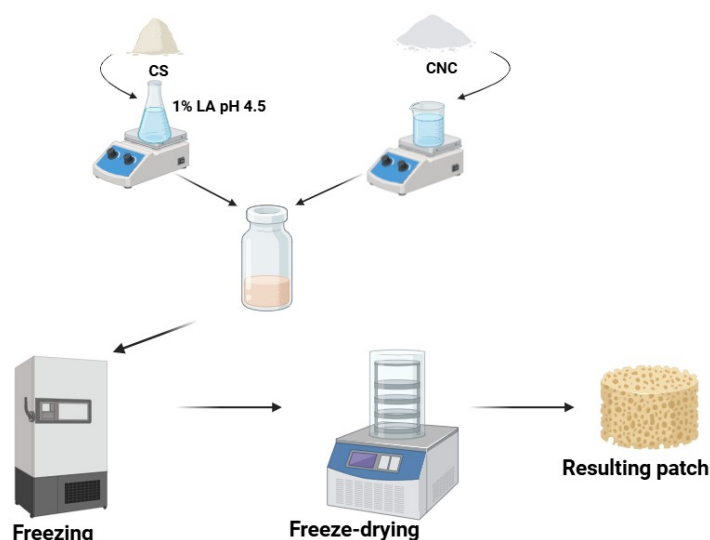


Figure 1. Schematic representation of the synthesis process of CS/CNC composite patches. The diagram illustrates the chemical cross-linking of chitosan (CS) with genipin (GP), the incorporation of cellulose nanocrystals (CNCs), and the two-step freezing and lyophilization process to generate the porous architecture.

CHARACTERIZATION OF THE FORMULATIONS

4.1. Swelling behaviour

The swelling behavior of the prepared chitosan (CS)-based sponges was evaluated *in vitro* to assess their hydrophilic properties and suitability for wound applications. Dried sponges were first equilibrated in a vacuum oven until a constant weight (W_0) was obtained. Subsequently, the samples were immersed in phosphate-buffered saline (PBS, pH 7.4) at room temperature and allowed to swell. At predetermined time points (1 min, 60 min, 360 min, and 1440 min), excess PBS on the surface was gently removed using absorbent paper, and the weight of the swollen sponges (W_a) was recorded.

The swelling ratio (SR, %) was calculated using the following equation:

$$SR(\%) = \frac{W_a - W_0}{W_0} \times 100\% \quad \text{Equation 1}$$

where W_0 - initial dry weight of the sponge, and W_a - the weight at swelling equilibrium.

The high porosity and hydrophilic character of the CS-based sponges promoted rapid water uptake and uniform swelling, suggesting their potential to maintain a moist environment favourable for wound healing, particularly in diabetic foot ulcers. All experiments were performed in triplicate ($n = 3$) to ensure reproducibility.

Mechanical properties

The mechanical properties of the hydrogels including the compressive modulus and the compressive strain at break point were evaluated using an Instron Universal Testing Machine 5565 (Instron, England) with a 100 N load cell and a crosshead speed of 2 mm.min⁻¹. Cylindrical hydrogel samples with a diameter of 25 mm and a height of 10 mm were used for the compression tests. Compressive modulus was defined as the slope of the linear region of the strain/stress curves, corresponding to 5-15 % strain. Data of the compressive strain correspond to the strain at the yield point. The results were reported as the mean $\pm$ standard error of at least seven specimens.

All tests were conducted under controlled laboratory conditions (25 °C $\pm$ 2 °C and 50 % relative humidity). Prior to testing, the samples were conditioned at 20 °C $\pm$ 2 °C and 65 % relative humidity until they reached constant moisture.

X-ray diffraction (XRD)

X-ray diffraction patterns were recorded in a diffractometer D5000 Siemens (Karlsruhe, Germany). The equipment operated at a voltage of 45 kV and a current of 30 mA and uses copper K-alpha (Cu K α) radiation with an average wavelength of λ (K α) = 0.15 nm (1.54 Å).

The aperture used was 0.6 mm. Diffractograms were registered in the angular region of 2θ from 5° to 40° , at room temperature, with a step time of 4 s and an angular increment of 0.005° .

Scanning electron microscopy

To evaluate the surface morphological structures, the sponges were sputter-coated with iridium using a Cressington 208HR high-resolution sputter coating (Cressington Scientific Instrument, UK). The surface morphologies were observed with a JSM 7200F field emission scanning electron microscope (Jeol, USA) at an accelerating voltage of 10 kV and 15 kV.

Cell culture

The embryonic fibroblast cell line (NIH 3T3) was cultured in Dulbecco's Modified Eagle Medium (DMEM) 4.5 g/L glucose (Merck KGaA, DEU) supplemented with 10 % fetal bovine serum (Merck KGaA, DEU), 2 % penicillin/streptomycin antibiotics and 2 % L-glutamine (Merck KGaA, DEU) in a CO₂ incubator under standard conditions (temperature 37 °C, 5 % CO₂, absolute humidity). For the viability assay, the cells were seeded at a density of 10×10^3 cells per well in 96-well tissue culture plates and cultured for 24 h.

MTT viability assay

Toxicity of the hydrogels was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay (8, 9). MTT is a yellow compound that when reduced by functioning mitochondria, produces purple formazan crystals that can be measured spectrophotometrically. The quantity of formazan generated by the mitochondrial metabolic activity is presumably directly proportional to the number of viable cells (10). For this, we prepared extracts of the sponges at 25 mg/mL in culture medium. The sponges were weighted and sterilized using ultraviolet light for 1 h and subsequently incubated with culture medium at 25 mg/mL in a

CO₂ incubator under standard conditions (temperature 37 °C, 5 % CO₂, absolute humidity) for 24 h. 3T3 fibroblasts were treated for 24 h with the extracts, and MTT 0.5 mg/mL was added 4 h before the expiry of this period. At the end of the treatment, a solubilization solution (10 % SDS solution in 0.01M HCl) was added to dissolve the formazan crystals. After overnight incubation at 37 °C, absorbance at 550 nm was measured in a microplate reader (MultiscanEX, Thermo Fisher Scientific, USA). Data are represented as percentage of control (untreated cells). One-way analysis of variance (ANOVA) test followed by Bonferroni's multiple comparison test was performed to analysis of data. A *p* value < 0.05 was considered as significant. Data of the cell assay are reported as the mean ± standard error of the mean of at least three independent experiments. Statistical analyses were performed with GraphPad Prism 9.3.1.

Antibacterial properties

Potential growth of pathogenic bacteria in the presence of the three different scaffolds was assayed. The five bacterial strains used for this purpose were *Staphylococcus aureus* (ATCC29213), *Escherichia coli* (ATCC25922), *Proteus mirabilis* (130941), *Klebsiella pneumoniae* (ATCC70063) and *Pseudomonas aeruginosa* (ATCC27853). Bacterial suspensions in Brain Heart Infusion broth adjusted to 0.5 McFarland standard ($\sim 1.5 \times 10^8$ CFU/mL) were directly applied (100 µL) onto the scaffolds, previously sterilized under UV radiation for 1 h, and incubated for 5 h at 37 °C. A bacterial suspension incubated without sponges was used as a control for any assayed strain. After this first incubation, bacterial counts were determined by plating on Columbia +5 % Sheep Blood Agar medium and further incubation at 37 °C, 24 hours. All cultures were carried out by triplicate. The evaluation of antibacterial activity was based on the determination of viable bacterial count after incubation in the presence of the scaffolds. Antibacterial activity was defined as bactericidal based on the

criterion of a reduction $> 3 \log_{10}$ CFU/mL (> 99.9 % killing) relative to the initial inoculum.

RESULTS AND DISCUSSION

Swelling and exudate absorption capacity

The swelling behavior of a scaffold is a fundamental property that dictates its performance in wound healing, particularly for chronic wounds like diabetic foot ulcers (DFU), which are characterized by high levels of exudate. An optimal swelling ratio ensures the efficient removal of excess fluid from the wound bed, preventing maceration of the periwound skin (11,12) while maintaining a moist environment conducive to cell migration.

In this study, the water uptake capacity of the lyophilized dressings was evaluated across 24 h (Fig. 2).

All formulations exhibited an immediate hydration response, reaching an equilibrium state within the first 60 min. The pristine chitosan matrix (CS/GP) showed an absorption capacity of approximately 1700 %, while the incorporation of 10 % CNCs led to a sharp increase in this capacity, reaching values of ~ 3300 %. This significant enhancement compared to the control is attributed to the high hydrophilicity of CNCs and a decrease in the crystallinity index of the chitosan matrix at low filler concentrations, as previously suggested by XRD analysis. This structural disruption increases the free volume within the scaffold, facilitating the diffusion of nutrients and oxygen essential for cell survival (13, 14).

Conversely, increasing the CNC content to 50 % led to a reduction in swelling (~ 2000 %) relative to the 10 % group. This behavior suggests that high CNC concentrations establish a rigid, highly crystalline framework that acts as a physical constraint, limiting the expansion of the polymeric network despite the inherent hydrophilicity of the components. These findings indicate that CNC incorporation allows for the precise tuning of the absorptive properties of CS-based scaffolds, ensuring structural integrity while

managing exudate levels, a critical factor for advanced wound dressing applications.

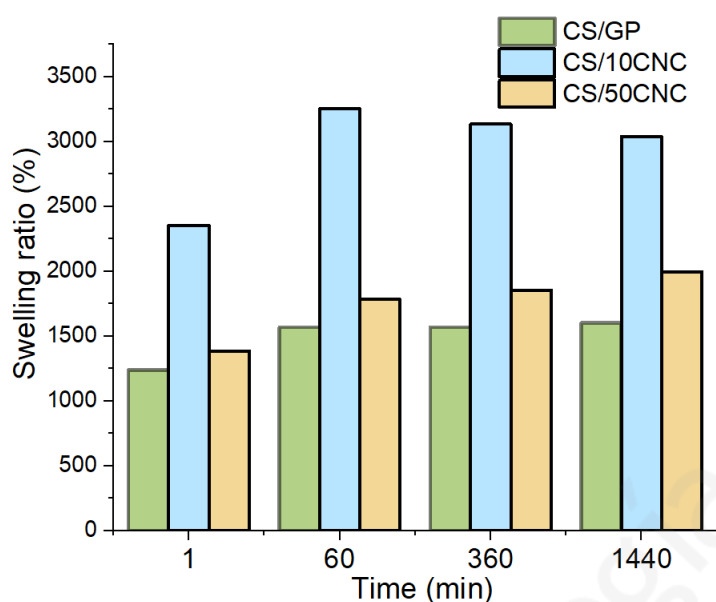


Figure 2. Swelling ratio (%) of CS/GP, CS/10CNC, and CS/50CNC sponges at 1 min, 60 min, 360 min, and 1440 min.

From a clinical perspective, exudate management in DFUs requires preventing both wound bed desiccation and periwound skin maceration. The synthesized nanocomposites address this through rapid vertical fluid absorption (reaching equilibrium within 60 min) and superior retention capacity (swelling ratios between 2000 % and 3300 %). Importantly, the mechanical robustness of the reinforced matrix (CS/50CNC) prevents structural collapse under compressive dressing loads, avoiding the unwanted release of absorbed fluids back onto the surrounding healthy margins. Although experimental Water Vapor Transmission Rate (WVTR) was not quantified in this study, representing a limitation to be addressed in subsequent preclinical validations, the open interconnected porous network observed by SEM is consistent with porous architectures that support physiological moisture vapor dissipation, which typically ranges between 2000 and 2500 g/m²/day in effective breathable wound dressings.

Mechanical properties

The macroscopic behavior of the sponges during mechanical testing is illustrated in figure 3. The patches exhibited a typical cylindrical geometry with an initial height of 15 mm (Fig. 3B). Upon the application of a compressive load in the mechanical tester (Fig. 3C), the scaffolds underwent a controlled deformation, reducing their height to 6 mm (Fig. 3A), which represents a 60 % vertical strain. Notably, even after reaching high deformation levels, the patches (Fig. 3D) remained cohesive and maintained their structural integrity without showing signs of fracture, crumbling, or permanent disintegration. This macroscopic robustness confirms the successful formation of a stable, interconnected porous network.

The ability of the patches to withstand large compressive deformations (from 15 mm to 6 mm) while remaining intact is a critical feature for wound healing applications (Fig. 3 A and D). In the clinical management of diabetic foot ulcers (DFU), scaffolds are often subjected to mechanical pressure from secondary dressings or the patient's own movement. A brittle material would fail under these conditions, potentially causing irritation or leaving debris in the wound bed.

The visual evidence of the patch's stability after compression (Fig. 3D) demonstrates that the CS/GP/CNC system is not only biomimetic in terms of stiffness but also highly conformable. This flexibility allows the sponge to adapt to irregular wound topographies, ensuring consistent contact with the wound bed—a factor that has been shown to facilitate cell migration and accelerate re-epithelialization. The synergy between this macroscopic 'sponge-like' behavior and the microscopic reinforcement from the CNCs creates a scaffold that is both protective and adaptable to the dynamic environment of a chronic wound.

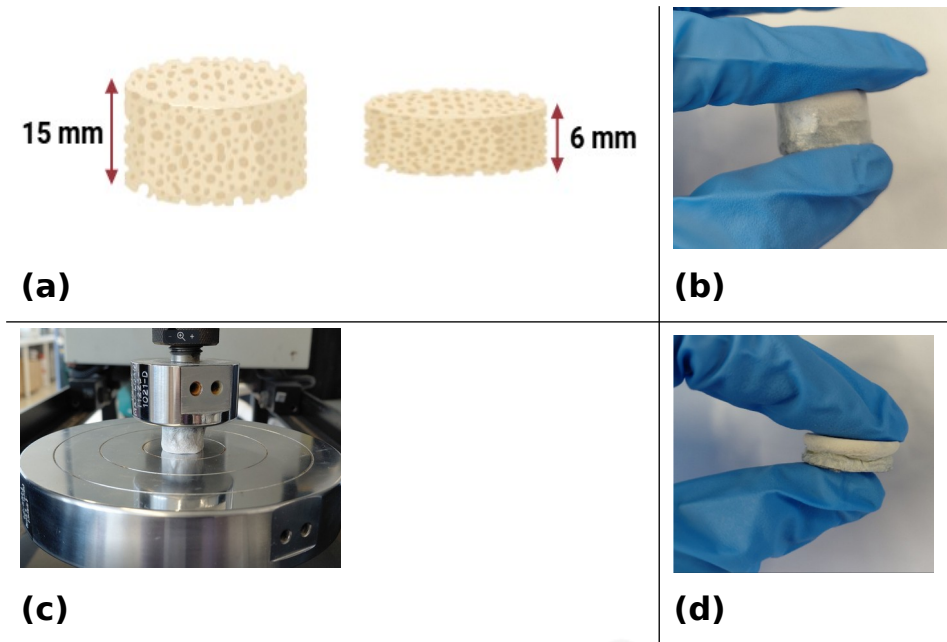


Figure 3. Macroscopic evaluation of mechanical stability under compression. A. Comparison of patch height before (15 mm) and after (6 mm) 60 % vertical strain. B. Photo of the pristine patch. C. Experimental setup during the compression test. D. Recovery of the scaffold after load removal, demonstrating structural integrity and lack of disintegration.

The mechanical behavior of the CS/GP/CNC sponges was evaluated through compression tests (Fig. 4). The stress-strain curves exhibited the characteristic three-stage profile of porous scaffolds: an initial linear elastic region, followed by a plateau related to pore wall collapse, and a final densification stage. The incorporation of CNCs significantly reinforced the polymeric matrix. The compressive modulus (E_c) increased from 0.08 ± 0.01 MPa for the pristine CS/GP to 0.15 ± 0.03 MPa for the 10 % CNC composite. Notably, the CS/50CNC sample reached an E_c of 0.45 ± 0.05 MPa, representing a 5.6-fold increase in stiffness compared to the control group. Furthermore, the maximum stress at 80 % strain rose from 0.35 MPa (CS/GP) to 1.40 MPa for the 50 % CNC formulation, demonstrating a substantial enhancement in structural load-bearing capacity.

The mechanical reinforcement provided by the CNCs is consistent with the structural findings from XRD analysis. At high concentrations (50 %), the CNCs form a rigid, interconnected crystalline network that restricts the mobility of the chitosan chains, effectively dissipating energy during compression. From a clinical perspective, these results are highly relevant for wound healing applications. Literature indicates that the elastic modulus of human dermis typically ranges from 0.1 to 2.0 MPa (5). While the pristine CS/GP hydrogel falls slightly below this range, the addition of CNCs allows the scaffold to reach a biomimetic stiffness (~ 0.45 MPa for CS/50CNC) that matches native tissue. This mechanical tuning is crucial for diabetic foot ulcer (DFU) treatment, as it ensures the patch can withstand the pressure of dressings and physical contact without losing its porous architecture, while remaining flexible enough to conform to irregular wound beds without inducing secondary mechanical trauma.

The rationale for selecting 10 % and 50 % CNC concentrations followed a boundary-testing design to establish the functional limits of the nanocomposite platform: a low-filler regime (10 %) aimed at maximizing free volume and fluid uptake (~ 3300 %), and a high-filler regime (50 %) intended to achieve the biomechanical threshold of human dermal tissue ($E_c \sim 0.45$ MPa). Nevertheless, we acknowledge that the absence of intermediate filler concentrations (e.g., 20–40 wt %) represents a study limitation that restricts the detailed mapping of the dose-property relationship. Systematic fine-tuning across intermediate ratios will be addressed in future formulation optimization phases.

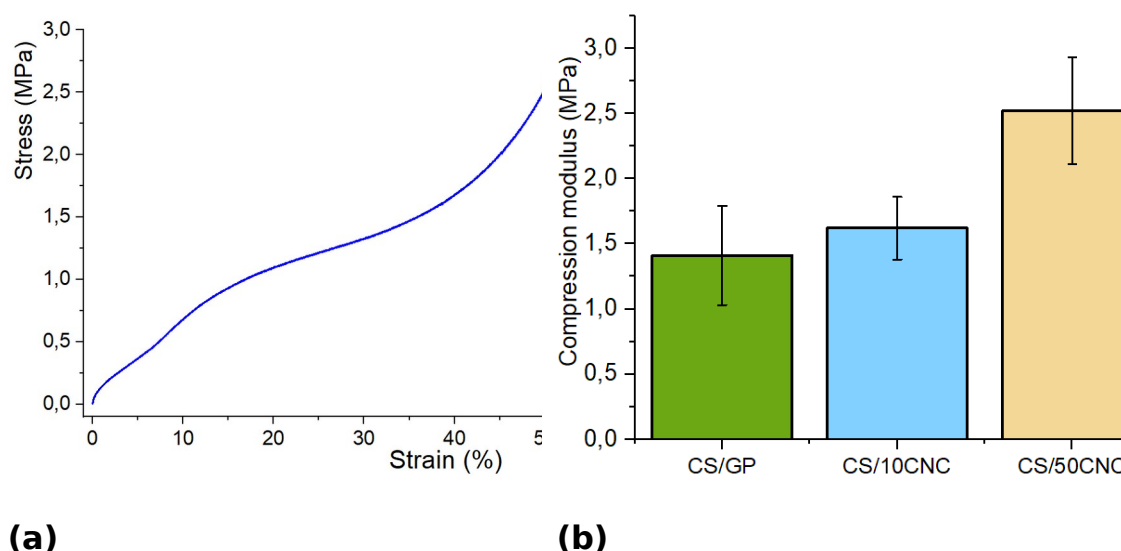


Figure 4. Mechanical characterization under compression. A. Representative stress-strain curves for the composite scaffolds. B. compressive modulus for CS/GP, CS/10CNC, and CS/50CNC formulations. Results are expressed as mean $\pm$ standard error ($n = 7$).

These results demonstrate that the scaffold containing 50 % CNC achieves biomimetic stiffness, falling precisely within the physiological range reported for the human dermis (0.1-2.0 MPa) (5). This "mechanical matching" is a critical feature for diabetic foot ulcer management, as it ensures that the device can withstand the dynamic stresses of patient ambulation without structural failure. Furthermore, the ability of the patch to recover its shape after significant deformation confirms that the internal porous architecture remains intact under load, which is essential for maintaining continuous gas exchange and supporting cell infiltration throughout the healing process.

X - ray diffraction (XRD)

The crystalline structure of the nanocomposites was investigated by XRD (Fig. 5). The pristine CS/GP hydrogel exhibited two broad diffraction halos at $2\theta \approx 10^\circ$ and 20° , characteristic of the semi-crystalline nature of chitosan and its amorphous regions induced by GP neutralization. Upon the addition of CNCs, the diffraction patterns underwent significant changes. For the 50 % CNC composite, sharp and intense reflections emerged at $2\theta \approx 15.5^\circ$ and 22.6° , corresponding to the (110) and (200) lattice planes of Cellulose I. The preservation of these sharp peaks suggests that the CNCs maintain their high crystallinity and intrinsic needle-like structure within the polymeric matrix. Furthermore, the suppression of the CS reflection at 10° in the nanocomposites suggests that the CNCs hinder the close packing of chitosan chains through the formation of an extensive interpenetrating hydrogen-bonding network. These structural arrangements are consistent with the improved mechanical integrity and cytocompatibility observed in the composite systems.

The crystallinity index (Crl) was determined using the Segal method to quantify the structural changes induced by CNC reinforcement. The CS/GP matrix exhibited a Crl of 31.5 %, typical of neutralized chitosan hydrogels. While the 10 % CNC addition resulted in a more disordered arrangement (Crl $\sim$ 17.1 %), the 50 % CNC composite showed a well-defined crystalline profile dominated by Cellulose I reflections. This structural reinforcement correlates with the cell viability results, as the highly organized crystalline domains of CNCs provide a more stable and biomimetic topography for fibroblast attachment and metabolic activity.

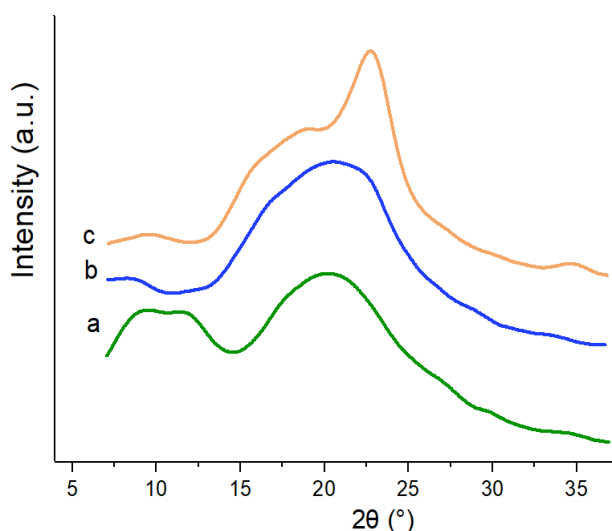


Figure 5. X-ray diffraction (XRD) patterns of the hydrogel systems: (a) CS/GP, (b) CS/10CNC, and (c) CS/50CNC. The characteristic reflections of Cellulose I at $2\theta \approx 15.5^\circ$ and 22.6° is highlighted, indicating the successful incorporation and preservation of CNC crystallinity within the matrix.

Morphological analysis (SEM)

The internal architecture of the lyophilized sponges was examined by SEM to evaluate the effect of CNC incorporation on the porous network (**Fig. 6**). All formulations exhibited a highly interconnected, three-dimensional 'honeycomb' morphology, which is characteristic of the ice-templating process during freeze-drying. The pristine CS/GP matrix displayed large, open pores defined by smooth and thin polymeric walls (Fig. 6 A and B).

Upon the addition of CNCs, a concentration-dependent structural evolution was observed. The CS/10CNC scaffolds maintained an open-cell structure similar to the control, although the pore walls showed a slight increase in surface roughness (Fig. 6 C and D). In contrast, the CS/50CNC sponges exhibited a more robust and compact architecture (Fig. 6 E and F). In these samples, the pore walls appeared significantly thicker and displayed a granular texture, suggesting the formation of a dense CNC-reinforced network within the chitosan

lamellae. Despite this reinforcement, the high degree of pore interconnectivity remained intact across all formulations.

The morphological features observed by SEM provide a structural basis for the physicochemical and biological performance of the nanocomposites. The maintenance of an interconnected porous network is fundamental for the high cell viability detected in the MTT assays (> 90 %), as it ensures the efficient diffusion of oxygen and nutrients throughout the scaffold volume.

The transition from the thin-walled structure of the CS/GP control to the reinforced, thicker walls of the 50 % CNC composite perfectly correlates with the mechanical and swelling data. The thinner walls in the 10 % CNC group allow for the massive structural expansion observed during hydration (~3300 %). Conversely, the thicker and more rigid walls in the 50 % CNC group—arising from the crystalline Cellulose I domains confirmed by XRD—provide the structural integrity necessary to reach a compressive modulus of 0.45 MPa. This reinforced architecture acts as a physical constraint that limits excessive swelling (~2000 %) while preventing pore collapse under external pressure, making the CS/50CNC system an ideal candidate for managing exudate in diabetic foot ulcers.

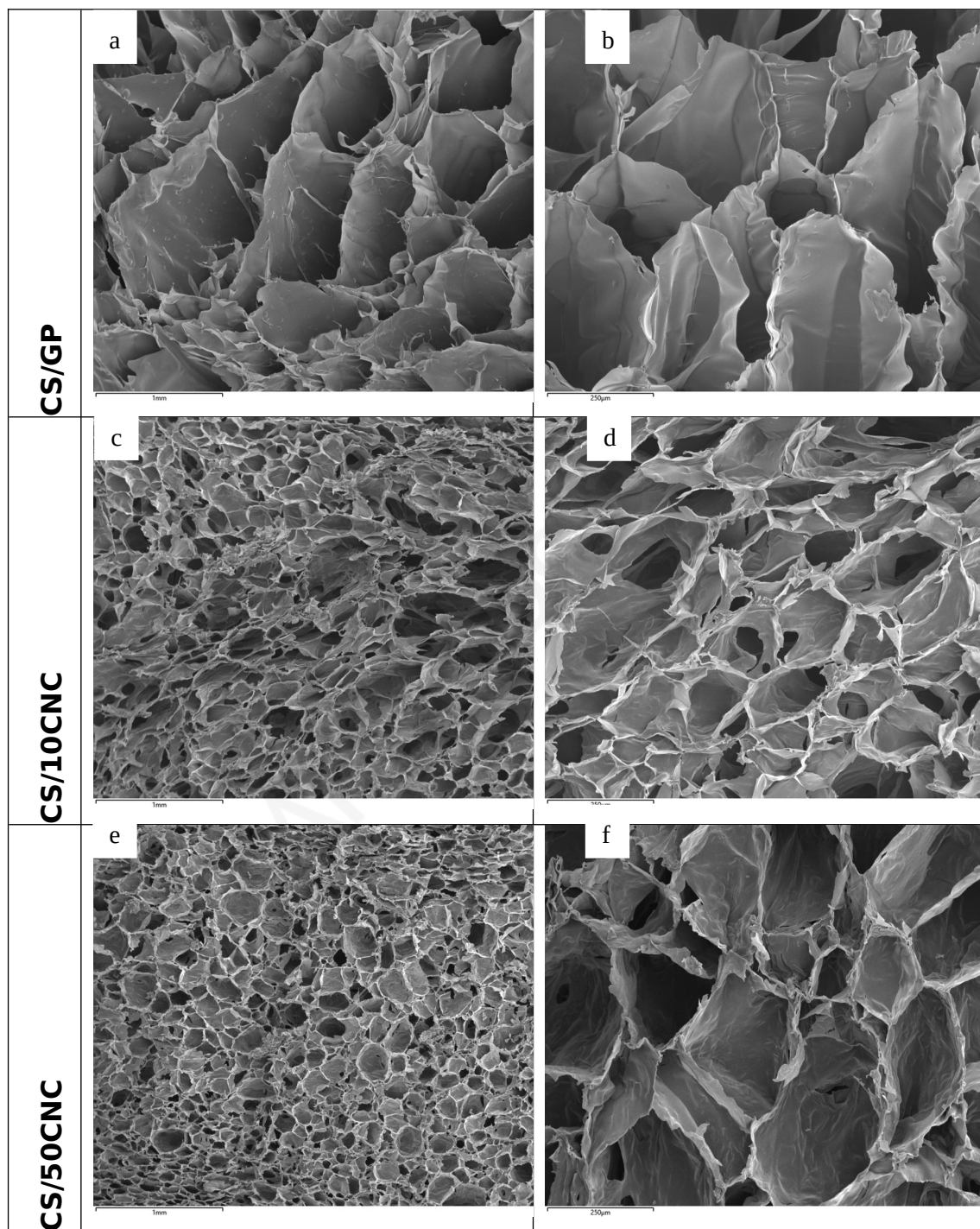


Figure 6. Scanning electron microscopy (SEM) micrographs of the cross-sectional morphology of sponges with varying CNC content (10 % and 50 % w/w). The left column displays low-magnification panoramic views (scale bar: 1 mm) illustrating the overall porous network. The right column presents high-magnification details of the

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pore wall architecture (scale bar: 250 μm). All formulations exhibit a highly interconnected, three-dimensional 'honeycomb' structure generated by the ice-templating process during lyophilization.

The highly interconnected porous network identified via SEM is fundamental for promoting angiogenesis (15,16) and ensuring efficient nutrient transport throughout the wound bed. The reinforced pore walls visible in the micrographs provide a structural basis for the material's superior mechanical performance; specifically, the crystalline CNC network serves as an internal nanoscaffold that stabilizes the overall patch architecture. This robust framework prevents pore collapse even when the scaffold is fully saturated with exudate, thereby ensuring that gas exchange and ulcer oxygenation remain uninterrupted during the clinical treatment.

In vitro cytocompatibility

The metabolic activity of 3T3 fibroblasts exposed to the conditioned media (25 mg/mL) was determined via MTT assay. As shown in figure 7, pristine CS exhibited significant cytotoxicity, with cell viability dropping to 38.1 % ($p < 0.0001$), well below the 70 % threshold established by ISO 10993-5. Interestingly, the formulation of CS/CNC nanocomposites effectively neutralized this effect. The CS/GP system showed a cell viability of $95.5 \% \pm 9.9 \%$. Furthermore, the integration of CNCs maintained high cytocompatibility levels, with values of $90.5 \% \pm 7.7 \%$ and $110.7 \% \pm 8.6 \%$ for 10 % and 50 % CNC content, respectively. No significant statistical differences were found between these nanocomposites and the negative control ($p > 0.05$). These findings suggest that the electrostatic interaction between CS, GP, and the CNC network mitigates the inherent toxicity of the cationic polymer, providing a safe microenvironment for cell growth.

Figure 7. Metabolic activity of 3T3 fibroblasts exposed to conditioned media (25 mg/mL) determined via MTT assay. Data are presented as mean $\pm$ SD ($n = 3$). Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test ($p < 0.0001$ relative to the control).

These findings are pivotal for ensuring patient safety. The selection of genipin as a naturally derived cross-linking agent, replacing traditional toxic synthetic alternatives, results in a dressing with negligible cytotoxicity. It is important to emphasize that the in vitro MTT assay on NIH 3T3 fibroblasts was conducted in accordance with ISO 10993-5 guidelines as an essential preliminary cytotoxicity screening, demonstrating that genipin cross-linking effectively abrogates the basal cytotoxicity of unreacted chitosan (raising cell viability from 38.1 % to >90 %). However, evaluating a standard embryonic murine fibroblast cell line under standard normoglycemic conditions represents an inherent limitation. The microenvironment of chronic diabetic foot ulcers is notoriously hostile, marked by sustained hyperglycemia, cellular senescence, excessive reactive oxygen species, and persistent pro-inflammatory macrophage infiltration. Therefore, while our data establish the baseline biosafety of the biomaterial, further investigations utilizing primary human dermal

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fibroblasts under hyperglycemic stress, scratch/migration wound-healing assays, inflammatory macrophage co-cultures (M1/M2 polarization), and diabetic in vivo wound models are imperative to comprehensively validate its regenerative efficacy.

Antibacterial properties

After incubation and the subsequent plate count agar method, the observed change in the number of viable bacteria cells was used as an indication of antibacterial properties of the scaffolds.

All assayed strains were severely affected, indicating biocidal activity of all scaffold formulations, since the final microbial counts (CFU/ml) were significantly lower than the inoculum (Fig. 8). However, the gram-positive bacteria assayed, *Staphylococcus aureus*, was less affected than the other four gram-negative species.

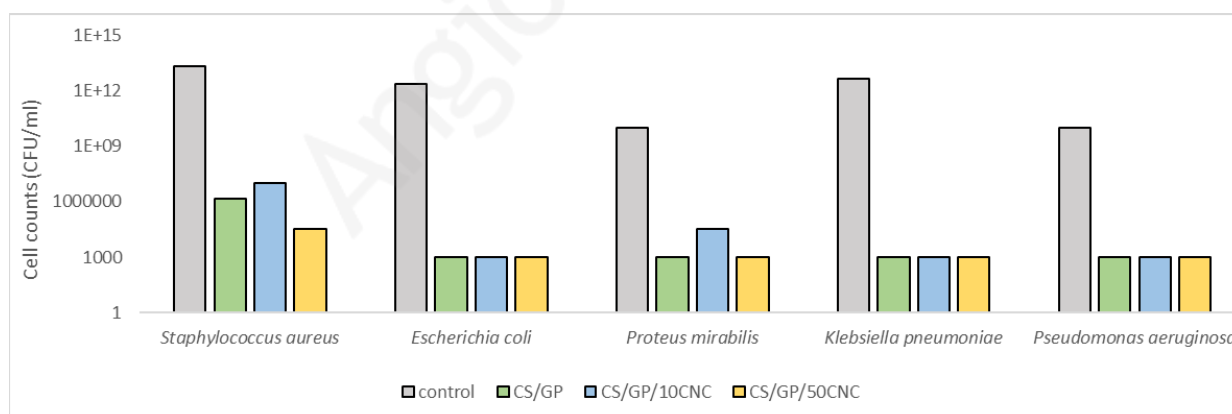


Figure 8. Antibacterial activity for CS/GP, CS/10CNC, and CS/50CNC formulations. Results are represented as the number of viable bacterial cell counts (CFU/ml). The control bar indicates the growth of the different strains without any scaffold. CFU: Colony-Forming Unit.

The pronounced reduction in viable counts—particularly evident against Gram-negative species (reductions $> 5 \log_{10}$)—is primarily

driven by the intrinsic polycationic nature of chitosan, whose protonated amino groups interact electrostatically with the negatively charged bacterial outer envelope, inducing membrane permeabilization and leakage of intracellular contents. While genipin cross-linking partially consumes primary amino groups, the high surface-to-volume ratio of the porous sponge maintains substantial contact-killing capability. However, the precise degree to which CNCs or genipin may exert synergistic or additive antibacterial effects was not independently decoupled in this assay, which constitutes a limitation to be addressed through mechanistic kinetic-kill curves in future works.

CONCLUSIONS

This study successfully developed and characterized novel bio-inspired nanocomposite scaffolds based on chitosan, genipin, and cellulose nanocrystals (CNCs) for the advanced treatment of diabetic foot ulcers. The incorporation of CNCs allowed for the precise tuning of the material's mechanical properties, where the formulation with 50 % CNC achieved a compressive modulus of 0.45 MPa, providing a mechanical match with the physiological range of human dermis and ensuring the patch can withstand the dynamic stresses of patient ambulation. Furthermore, these lyophilized dressings demonstrated an exceptional fluid absorption capacity of up to ~3300 %, critical for preventing periwound maceration while maintaining a controlled moist environment. SEM and XRD analysis confirmed a highly interconnected honeycomb-like architecture reinforced by a crystalline CNC network that remained stable under physiological conditions, preventing pore collapse and ensuring oxygen transport. The use of genipin as a natural cross-linker resulted in a system with negligible cytotoxicity, providing a safe microenvironment for tissue regeneration. Additionally, all formulations showed bactericidal activity, particularly against gram-negative bacteria. In conclusion, these synthesized CS/GP/CNC nanocomposites represent a promising

therapeutic tool in the field of Angiology and Vascular Surgery, with the potential to optimize the healing process and significantly reduce infectious complications in patients with diabetic foot ulcers.

Angiología

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