Nanostructured scaffold with biomimetic and antibacterial properties for wound healing produced by ‘Green electrospinning approach’

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ABBREVIATIONS

3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT); Dulbecco’s Modified Eagle Medium (DMEM); extracellular matrix (ECM); Energy dispersive X-ray spectrometer (EDS); fetal bovine serum (FBS); Fourier transform infrared-attenuated total reflectance spectroscopy (FTIR-ATR); gelatin (GL); gentamicin sulphate (GS); (3-glycidyloxypropyl)trimethoxysilane (GPTMS); neonatal normal human dermal fibroblasts (NHDF-Neo); silver (Ag); scanning electron microscopy (SEM); silver nanoparticles (AgNPs); transmission electron microscopy (TEM); Young’s modulus (E)

ABSTRACT

Hypothesis. Wound healing is a complex process that often requires treatment with antibacterial agents to avoid infection, which affects the optimal tissue regeneration process. Ideal scaffolds for wound healing treatment should combine biomimetic features to ensure the tissue growth on properly designed extracellular matrix (ECM)-like scaffolds and antibacterial properties in order to avoid bacterial colonization.

Experiments. In this work, gelatin cross-linked nanofibers (GL-nanofibres), with diameters ranging from 200 to 300 nm, were prepared via a “green electrospinning technique” to mimic the structure and composition of the extracellular matrix (ECM), and promote the normal skin wound healing process. Nanofibres were doped with two antibacterial agents (gentamicin sulphate or silver nanoparticles) to achieve an antibacterial effect against Gram+ and Gram- bacteria.

Findings. The ECM-mimicking structure of GL-nanofibres was not affected by the presence of the antibacterial agents, which were homogeneously distributed within the mats as shown by SEM and EDS. The antibacterial properties of the developed matrices were confirmed using 4 strains (*S. aureus*, *S. epidermidis*, *P. aeruginosa* and *E. coli*) while the biocompatibility of the developed substrates and their ability to induce cell growth was assessed using Neonatal Normal Human Dermal Fibroblasts (NHDF-Neo).

KEYWORDS: antibacterial nanofibers, electrospinning, ECM-like scaffolds, gentamicin, silver nanoparticles, gelatin, wound healing

1. Introduction

Wound healing is a dynamic, interactive, multicellular process involving extracellular matrix (ECM), cytokines, blood cells, parenchymal cells, etc [1]. During wound healing, five overlapping stages occur: hemostasis, inflammation, proliferation, contraction, and remodelling [2]. The prevention of wound infection during this process is a major challenge that needs a solution urgently, because high microbial colonization at the wound site delays the wound healing and in long-term it can lead to life-threatening conditions [3]. In this attempt, wound care dressings have been largely investigated with the aim to maintain favourable conditions for the healing process and to protect the wound from environmental threats and penetration of bacteria. Different materials, such as cotton, honey, animal fats, and vegetables fibres, have been used for wound dressings [4, 5]. However, these conventional treatments may have many drawbacks such as strong adherence to the wound, need of frequent replace and presence of microorganisms or chemicals which might be detrimental to a proper wound healing. Therefore, electrospun polymer mats have emerged for their great potential as wound dressing materials due to their close structural resemblance to the ECM (which enhances the tissue growth), their high porosity that allows gas permeation, and the large surface to volume ratio which provides an open structure for drainage of exacerbated exudate and hemostasis enhancement [6]. Hence, biodegradable polymer nanofibres with different chemical and physical properties using both - synthetic polymers such as polyurethanes [7-9], poly(caprolactones) [10, 11] and poly(vinyl alcohols) [10, 12, 13] and natural polymers such as collagen [14], gelatin [7, 15], silk fibroin [16, 17] and chitosan [16, 18] have been applied for wound healing having bioresorption as major advantage compared to non-degradable polymers.

Among natural polymers, gelatin (GL) is a protein derived from partial hydrolysis of native collagens, which are the most abundant structural proteins found in the animal body on skin, tendons, cartilage and bones [19]. GL has been widely used for engineering tissues, dressings for wound healing, and drug delivery because of its similarity, as adhesive protein, to the more expensive collagen [20-23]. GL has been also found to provide hemostasis and promote cell adhesion and proliferation during wound healing [24]. However, poor mechanical properties and low thermal stability, limits the biomedical application of this material which can be improved by cross-linking [25-27] and/or combining with other more stable polymers [7, 21, 28]. Research efforts for further improvement of GL nanofibrous matrices have been mainly targeting functionalization with pharmaceutical [29, 30] and bioactive agents [30-35] as well as drug release modulation. Incorporated drugs and proteins play an active role in the wound healing process, either directly or indirectly, as cleansing or debriding substances to remove necrotic tissue as antibacterial agent to prevent or treat infections, or as bioactive molecules (e.g. growth factors) to aid tissue regeneration. Moreover, the development of medicated dressing aims to prolong the action of active agents over time by allowing sustained release from a nanofibrous polymeric matrix without the need for frequent dressing change, depending on the severity of the wound [36].

In the current work, GL nanofibrous matrices loaded with the antibacterial drug gentamicin sulphate (GS) or silver nanoparticles (AgNPs), were fabricated to induce a potent antibacterial effect against Gram+ and Gram- bacteria and to guarantee a controlled release of the antibacterial during time and space via the high surface area to volume ratio of the nanofibre system during tissue healing process. Furthermore, the topical local delivery from a bioresorbable nanofibrous matrix may reduce the risk associated to over toxicity and systemic toxicity targeting only the infection site [37]. GS and AgNPs were selected due to their antibacterial activity against both

Gram+ and Gram- bacteria [38]. GS is a widely used aminoglycoside antibiotic. It is heat-stable, making it an ideal candidate for a range of fabrication and incorporation methods, which require temperatures higher than the physiological condition (about 37 °C). Silver (Ag) has been commonly applied for the treatment of burns and chronic wounds as an alternative to antibiotics, since the cases of bacterial resistance to currently used antibiotics has been increasing. Dressings designed to elute silver ions have been shown to be effective at reducing bacterial colonisation of wounds [19, 39, 40] as ointment and cream (silver sulfadiazine) [41], or it can be loaded in matrices as nanoparticles for its controlled release [42, 43]. Several products incorporate silver for use as a topical antibacterial agent, such as silver nitrate, silver sulphadiazine (Flammazine™) [44], silver sulphadiazine/chlorhexidine (Silverex®), and silver sulphadiazine-impregnated lipidocolloid wound dressing Urgotul SSD® [40, 45]. Further, AgNPs show more efficient antibacterial properties than other salts due to their extremely large surface area, which provides better contact with microorganisms [46, 47].

The GL crosslinked nanofibres doped with antibacterial agents were obtained using an environmental and cell friendly method (“green electrospinning”) as only water was used as solvent within all the process. The fabrication process was set up in order to crosslink the GL nanofibers within the fabrication process avoiding a second step which could affect the nanofibrous mat morphology and consequently its biomimetic properties.

Developed nanofibers were analysed for their physicochemical and morphological properties by scanning electron microscopy (SEM) and Energy dispersive X-ray spectrometer (EDS), transmission electron microscopy (TEM) and their mechanical properties were evaluated by tensile tests. GS release from GL nanofibres membranes was evaluated by means of UV-VIS spectroscopy, whilst the AgNPs release was indirectly investigated from the antibacterial test

results conducted *in vitro* against four different bacterial species. Finally, Neonatal Normal Human Dermal Fibroblasts (NHDF-Neo) were seeded on the surface of electrospun membranes in order to test the cytocompatibility and explore their further application in tissue engineering.

2. Materials and methods

2.1 Materials

GL (type A from porcine skin), (3-Glycidyloxypropyl)trimethoxysilane (GPTMS), GS and silver nitrate were supplied by Sigma Aldrich.

2.2 Sample preparation

2.2.1 Preparation of solutions for electrospinning

The GL solutions with improved antibacterial properties, coded as GL/GS and GL/AgNPs according to the antibacterial agent incorporated, were prepared as follows:

- GL/AgNPs: 2.5% or 5% w/w AgNO₃ (based on the weight of the GL powder) were dissolved in demineralised water, followed by GL addition to the solution (15% wt/v). The AgNO₃-containing GL solution was aged in an amber glass bottle while being stirred at 50°C for 18 h. The reduction of Ag⁺ ions into elemental Ag (i.e., nAg) in the AgNO₃-containing GL solutions was visualized by changes in the color of the solutions: from light yellow of the GL solution to brown of the 18 h-aged AgNO₃-containing solution (reported as Supporting Information, Fig. S1). In this work an ageing time of 18h was selected to achieve AgNP formation avoiding GL hydrolytic degradation. As previously reported by Rujitanaroj et al., the amount of AgNP

formed by AgNO₃ reduction increased monotonically with increasing aging time until 10 days. However, the GL hydrolytic degradation occurred for a mixing period longer than 2 days [15]. - GS loaded solutions (GL/GS) were prepared by dissolving GL (15% wt/v) and GS in distilled water at 50°C and mixed together to obtain blends with weight ratios of GS to GL of 2.5, 5, 7.5 and 10% w/w. Solutions were left under magnetic stirring at 50°C for about 40 minutes. GL solution without the addition of antibacterial agents was prepared following the procedures described above and used as control. Finally, an appropriate amount of GPTMS crosslinker was added to GL/AgNO₃, GL/GS and GL solutions and mixed for 1 h before spinning. The amount of GPTMS was calculated with respect to the molar concentration of amino groups in hydroxylysine, lysine and arginine residues of GL to obtain a molar ratio of 2:1 between the GL amino groups and the GPTMS molecules according to literature [48-50].

2.2.2 Electrospinning of GL nanofibres

Nanofibrous matrices were prepared by the electrospinning using a flat aluminium plate to collect randomly oriented nanofibres. The electrospinning system used (Biomedical Components s.r.l) for fibre preparation consists of a high voltage generator (PS/EL30R01.5-22 Glassman High Voltage); a volumetric pump (KDS210 of KD Scientific); a mobile syringe support and a collector as previously described [49]. Spinning parameters were set as voltage of 30 kV and a flow rate of 15 µl/min. Furthermore, the electrospinning apparatus was placed into a temperature-controlled chamber and the temperature was set at 50°C to avoid GL solution gelation within the needle during the electrospinning process. After spinning, all CS nanofibres were peeled off from the collector and characterized.

2.3 Morphological characterization through electron microscopy

2.3.1 Scanning electron microscopy (SEM) equipped with an energy dispersive X-ray spectrometer (EDS)

The fibre morphology of GL loaded nanofibres was observed by scanning electron microscopy (SEM LEO – 1430, Zeiss) at 20 kV. Using EDS, qualitative compositional analysis was performed and punctual elemental composition of materials was accomplished. Samples were sputter coated with gold in an under vacuum chamber prior to SEM-EDS examination. SEM micrographs were then analysed through ImageJ1.44g software. Fibre diameters were measured on three different SEM micrographs (50 measures were taken for each image) and reported as average value $\pm$ standard deviation.

2.3.2 Transmission electron microscopy (TEM)

The silver nanoparticles formation and incorporation into GL based nanofibres were determined using a Libra 120 TEM (Zeiss). TEM samples were prepared by dispersing small bundles of fibres in ethanol using an ultrasonic bath for 10 minutes. Then, the obtained solutions were deposited on a 300 mesh carbon coated TEM grid.

2.4 Fourier transform infrared-attenuated total reflectance spectroscopy (FTIR-ATR)

Chemical analysis of GL, GL/AgNPs and GL/GS nanofibrous matrices was performed by FTIR-ATR spectroscopy (Perkin-Elmer) over a range of 4000 cm^{-1} – 600 cm^{-1} at a resolution of 4 cm^{-1} and 16 scans.

2.5 Static water contact angle

Static contact angles of water on the nanofibre nonwoven surfaces were measured using the sessile drop method with a KSV instrument. Five μl of deionized water was dropped onto dry samples in air and the contact angle was calculated using a CAM200 Software. For each angle reported, at least five measurements on different surface locations were measured and results were expressed as average value $\pm$ standard deviation. As GL is a swellable material the contact angle value was collected immediately after drop deposition to avoid water adsorption.

2.6 Mechanical properties

The tensile mechanical properties were evaluated on nanofibrous membranes in dry condition by using a MTS QTest/10 device equipped with a load cell of 10 N. Rectangular strips of 20 mm $\times$ 10 mm size were cut from each sample and strained to break at a constant crosshead speed of 2 mm/min. Sample thickness was measured by using a Vernier digital calibre, obtaining values in the range of 110–140 μm . Using the associated software Test Works, break stress and strain were determined. The Young's modulus (E), ultimate tensile strength (UTS) and strain at failure (ϵ failure) were calculated from the stress–strain curve. Five specimens for each membrane were tested and results were expressed as average values $\pm$ standard deviation.

2.7 Antibacterial agent release quantification

GS release from GL/GS nanofibrous membranes were carried out by UV–VIS spectroscopy (CARY 500 SCAN UV–VIS–NIR Spectrophotometer). Samples were immersed in 3 ml of PBS

(pH 7.4) and GS concentration in the incubation media was measured after 1, 3, 6, 9 and 24 hours. The GS release concentration was reported as a percentage respect to the initial concentration and it was calculated from the absorption values using the calibration curves that were prepared starting from GS solutions of known concentrations. UV spectra were recorded in a range of 4000–600 nm.

On the other hand, Ag⁺ release was quantified by means of Inductively Coupled Plasma Atomic Emission Spectrometry Technique (ICP-AES) (ICP-MS, Thermoscientific, ICAP Q), after appropriate dilutions. Two ml of milliQ water were added to 4 mg of each sample and supernatants were collected after 1, 6, 24 and 168 hours.

Five measures for sample were used and the data was reported as mean value $\pm$ standard deviation.

2.8 Antibacterial activity of GL/GS and GL/AgNPs nanofibres

2.8.1 Disk diffusion method

Diffusion method was selected as screening assay to verify the antibacterial effectiveness of different types of electrospun nanocomposite fibrous matrices. Antibacterial activity of GL, GL/AgNPs, GL/GS nanofibrous membranes (5 mm sides squares previously sterilized by 30 minutes UV treatment) was tested against Gram-positive *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228) and Gram-negative *Pseudomonas aeruginosa* (ATCC 10145), *Escherichia coli* (ATCC 25922) based on EUCAST guidelines [51], with some modifications. Briefly, Mueller-Hinton agar was used as bacterial growth medium. The agar surface was inoculated by using a swab dipped in a cell suspension of a 0.5 McFarland turbidity standard, approximately corresponding to $1-2 \cdot 10^8$ Colony Forming Unit per ml (CFU/ml). All nanofibrous membranes were placed on each *S. aureus*, *S. epidermidis*, *P. aeruginosa* and *E. coli*

inoculated agar plate and incubated at 37 °C for 24 hours. Afterwards, the inhibition zone diameters were measured.

2.8.2 MTT colorimetric assay

The cytotoxicity of GS and AgNPs loaded nanofibres was assessed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)-based colorimetric assay [52]. Bacterial suspensions at a concentration of 5×10^5 CFU/ml were prepared in Tryptic Soy Broth (TSB) for *S. aureus*, *S. epidermidis*, *P. aeruginosa* and in Luria Bertani Broth for *E. coli*. Afterwards, 1 ml of each of these suspensions was used to dip 2 mg of GL, GL/AgNPs, GL/GS nanofibrous membranes (previously sterilized by 30 minutes UV treatment in 5 ml tubes) and samples were incubated at 37° C for 24, 48 and 72 hours at 100 rpm. At the end of each incubation time, bacteria were harvested by centrifugation at 12000 rpm for 10 minutes and incubated for 30 minutes in 1 ml of MTT working solution [0.03 g of MTT (Sigma-Aldrich) in 9.85 ml of PBS supplemented with 50 µl of a 20% glucose solution and 100 µl of a 1 mM menadione solution]. Bacteria were then harvested by centrifugation at 12000 rpm for 10 minutes and resuspended in 1 ml of DMSO:glycine 0.1 M pH 10.2 (7:1) buffer. Finally, absorbance of each sample was measured by spectrophotometric reading at 570 nm wavelength. The percentage of inhibition of GL/AgNPs, GL/GS samples, compared to GL membranes (controls), was determined as:

$$[1 - (\text{Abs}_{\text{treat}} / \text{Abs}_{\text{ctrl}})] \times 100 \quad (1)$$

Where:

$\text{Abs}_{\text{treat}}$; optical density of treated samples

Abs_{ctrl} ; optical density of controls.

2.9 Biological tests

Neonatal Normal Human Dermal Fibroblasts (NHDF-Neo) were purchased from Lonza Biosciences (Switzerland) and cultured as recommended by the seller. Briefly, fibroblasts were grown at 37 °C, 5% CO₂, in Dulbecco's Modified Eagle Medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and a 1% antibiotic mixture containing penicillin and streptomycin (100 U mL⁻¹).

In order to perform biocompatibility assays, the electrospun membranes (15 mm diameter) were mounted in 24-multiwell cell crowns (Scaffdex, Finland) and UV-sterilized for 15 min. A suspension of 1.5 x 10⁴ cells in DMEM was seeded dropwise on the top surface of the membranes and incubated at 37 °C, 5% CO₂ for 30 min. Then, fresh DMEM was added up to 1 mL volume.

2.9.1 Cytocompatibility studies

Cell viability was assessed with the live/dead staining (LIVE/DEAD® Cell Imaging Kit, Life Technologies, Thermo Scientific, USA) at 24 and 48 hours. According to the manufacturer's protocol, membranes were washed with phosphate buffered saline (PBS, Sigma-Aldrich, UK) and stained with 150 µL solution of 4 µM Ethidium homodimer-1 and 2 µM calcein in PBS. After 35 minutes of incubation at room temperature, cells were imaged with a Leica DM/LB fluorescence microscope to detect calcein (ex/em 488 nm/515 nm) and Ethidium homodimer-1 (ex/em 570 nm/602 nm), respectively.

Furthermore, the Presto Blue assay was exploited to test the metabolic activity of cells seeded on the different nanofibrous mats after 1 and 4 days of culture. A LS-50B Luminescence Spectrometer (Perkin Elmer, Waltham, MA) was used to measure the fluorescence (560nm excitation and 590nm emission) after 1.30 h of incubation with a 10% aliquot of Presto Blue (Thermo Scientific, USA). Results were expressed as mean ± standard deviation.

Finally, the cell morphology was observed by nucleus and cytoskeleton staining. Briefly, cells were fixed with 4% paraformaldehyde solution for 15 minutes, followed by three washing steps with PBS. Cells were then permeabilised using 0.1% v/v Tween20® in PBS for 5 minutes. Rhodamine-phalloidin was prepared using 1:100 dilution of phalloidin-tetramethylrhodamine B isothiocyanate (Sigma Aldrich, P1951) in 1% v/v Tween20® in PBS for 30 min, and then washed three times with PBS. One drop of DAPI (VECTASHIELD®) antifade mounting media was added to each sample, then covered with a glass slide and imaged using a Leica DM/LB fluorescence microscope.

2.10 Statistical analysis

Experiments were repeated three times and results expressed as a mean $\pm$ standard deviation. Statistical significance was calculated using analysis of variance (ANOVA). A comparison between two means was analyzed using Tukey's test with statistical significance level set at * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

3. Results

3.1 Morphological characterization through electron microscopy

GL based randomly oriented fibres with an average fibre dimension of 220 nm were fabricated as previously reported [49]. Fig. 1 reports SEM images of electrospun nanofibers of GL containing different concentrations of GS. All GS loaded nanofibres scaffolds exhibited smooth, uniform and bead-less fibrous randomly interconnected structure. No drug crystals were detected by the electron microscopy on the surface or outside the fibres loaded with the antibiotic agent, demonstrating the homogeneous character of the drug/polymer solution (Supporting Information,

Fig. S2). The corresponding size distributions of the developed nanofibres are listed in Fig. 1 (right side) and a uniform distribution of fibre diameter was observed with an average diameter of 217 ± 7 , 206 ± 7 , 184 ± 5 , 208 ± 5 and 216 ± 7 nm for pure GL, GL/GS2.5, GL/GS5, GL/GS7.5 and GL/GS10, respectively. A slight decrease in the fibre diameter was detected increasing GS concentration (except for GS amount equal to 10% w/w). This effect has to be ascribed to the incorporation of positively charged GS into the electrospinning solution, which may significantly increase the surface charge density of the spinning jet [53]. EDS analysis was performed on GL, GL/GS2.5, GL/GS5, GL/GS7.5 and GS/GS10 nanofibrous membranes to evaluate the distribution of GS in the electrospun samples. Inserts in Fig.1 (A, B, C, D and E) report the EDS element-mapping on the surfaces: the green dots, corresponding to the presence of Sulfur-containing species, were uniformly dispersed on the nanofibrous surfaces of all GS loaded samples. EDS element-mapping also detected the presence of Carbon and Oxygen elements characteristic of GL and peaks corresponding to Silicon containing compounds associated to the presence of GPTMS (data not shown).

Fig. 2 reports selected SEM images of electrospun nanofibrous matrices from both the 18 h-aged AgNO_3 -containing gelatin solutions (AgNO_3 2.5 and 5 % wt/wt). Cross-sectionally round fibres, without the presence of beads, were observed. The diameters of the AgNPs2.5 and AgNPs5 loaded nanofibres obtained from these solutions were 314 ± 12 and 277 ± 8 nm, respectively. The fibre diameter increased when AgNO_3 was added. Inserts on the top in Fig. 2 shows TEM images of nanofibrous matrices from the 18 h-aged AgNO_3 -containing gelatin solutions: AgNPs were found to be homogeneously distributed throughout the fibres, as also confirmed by the EDS pattern of elemental Ag (insert on the bottom) with the diameters of these particles being 13 ± 4 nm. Quantitative assessment by EDS of the amount of elemental Ag in fibre matrices indicated an

325 augmentation in value from 0.06 to 0.12 wt% by increasing the AgNO_3 amount from 2.5 to 5 %
 326 wt/wt.

327
 328 **Fig.1 Representative SEM micrographs, fibre distribution and EDS mapping of the**
 329 **electrospun nanofibres loaded with various GS amounts (green dots correspond to the presence**
 330 **of Sulfur-containing species). Notes: A) GL, B) GL/GS2.5, C) GL/GS5, D) GL/GS7.5 and E)**
 331 **GL/GS10.**

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334 **Fig. 2. Representative SEM images, TEM images and EDS mapping (red dots were**
335 **associated to Ag) of GL/AgNPs2.5 (A) and GL/AgNPs5 fibres**

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337 **3.2 Fourier transform infrared-attenuated total reflectance spectroscopy (FTIR-ATR)**

338 FTIR-ATR measurements were conducted on GS GL/GS nanofibres and GL/AgNPs to determine
339 if the introduction of GS or AgNPs formation of the electrospun nanofibres may affect the primary
340 gelatin structure and the GPTMS crosslinking mechanism. All spectra showed an amide I peak (C
341 = O stretch) at 1637 cm⁻¹, amide II peak (N-H bend) at 1542 cm⁻¹ and amide A peak (N-H
342 stretching vibration) at 3292 cm⁻¹, which are the distinguishing features of gelatin. Absorption
343 bands at 919 cm⁻¹ (Si-OH stretch), at 1031 cm⁻¹ (Si-O-Si stretch), at 1160 cm⁻¹ (Si-OH stretch)
344 confirmed the successful GPTMS crosslinking of GL which was not affected by GS or AgNO₃
345 addition to GL solutions (reported as Supporting Information, Fig. S3).

346

347 **3.3 Static water contact angle**

Interfacial phenomena on the GL based nanofibrous matrices were characterized by static water contact angle measurements to examine their wetting characteristics (Table 1). The electrospun GL membranes displayed a hydrophilic behavior with contact angles of about 60°. The addition of GS to GL solutions did not change the surface wettability significantly, except for GL/GS10 (*p<0.05). The enhanced hydrophilicity caused by the addition of GS10 is attributed to the hydrophilic behavior of the antibiotic agent, which is more consistent for GL/GS electrospun nanofibres obtained using an amount of GS equal to 10% wt/v. On the contrary, a significant decrease in the surface hydrophilicity of the GL based nanofibres was observed by increasing the initial AgNO₃ amount from 2.5 to 5 wt.% (*p<0.05) and it was probably due to the addition of hydrophobic metallic silver to the electrospun samples.

Table 1. Static water contact angle of GL, GL/GS2.5, GL/GS5, GL/GS7.5, GL/GS10, GL/AgNPs2.5 and GL/AgNPs5 electrospun nanofibres. Data are reported as average values and the standard deviations. Statistical analysis was reported compared to GL nanofibers (*p<0.05).

Sample	Static water contact angle (°)
GL	60.6±1.8
GL/GS2.5	61.7±5.3
GL/GS5	62.0±6.5
GL/GS7.5	60.1±4.0
GL/GS10	51.9±1.8*
GL/AgNPs2.5	67.0±2.5*
GL/AgNPs5	70.1±2.2*

3.4 Mechanical properties

Tensile tests were carried out on GL electrospun nanofibres in dry conditions to determine the effect of both antibacterial agents on sample stiffness. All samples had a uniform thickness of about 100-150 μm . GL nanofibrous matrices showed an elasto-plastic behaviour; at low strains (lower than 4%) the stress increased linearly with an increase in the strain, while for strains $>4\%$ the stress increased slowly with increasing strain until failure occurred (reported as Supporting Information, Fig. S4). Table 2 shows the elastic moduli (calculated from the slope of the linear portion of the stress–strain curve, the ultimate tensile strength (UTS) and the strain at failure ($\epsilon_{\text{failure}}$) of the GL nanofibres tested in dry state. The introduction of GS or AgNO_3 in GL solutions significantly increased the Young's modulus (E) and the UTS of GL electrospun matrices loaded with an antibacterial agent compared to bare GL nanofibres. Moreover, as the blending ratio of GS or AgNO_3 increased, the Young's modulus and UTS became higher. The Young's modulus and the UTS of membranes containing AgNO_3 5 wt.% was significantly different from GL/ $\text{AgNP}2.5$ and GL (** $p<0.01$), confirming that the reinforcing effect of the nanoparticles in the polymeric nanofibrous matrix is consistent with previously reported results [56]. A significant increase of the Young's modulus was also observed for GS loaded samples compared to control (* $p<0.05$). The reinforcement features of the GS-doped nanofibres can be ascribed to the homogenous distribution of GS confirmed by EDS analysis which excluded the formation of GS aggregates as well as to the nanofibres fabrication process which loaded GS into the nanofibres bulk.

A reduction of $\epsilon_{\text{failure}}$ was measured when the amount of antibacterial agent increased suggesting improvement of the rigid behavior of the antibacterial nanofibres compared to bare GL nanofibres.

Table 2. Elastic modulus calculated from the elastic region of the corresponding stress-strain curves of wet GL-based nanofibrous matrices (average value $\pm$ standard deviation), * $p < 0.05$ and ** $p < 0.01$.

Sample	E(MPa)	UTS (MPa)	ϵ failure (%)
GL	6.1 $\pm$ 2.7	0.26 $\pm$ 0.07	7.5 $\pm$ 1.2
GL/GS2.5	10.4 $\pm$ 1.7*	0.39 $\pm$ 0.08	7.2 $\pm$ 1.1
GL/GS5	12.6 $\pm$ 2.0*	0.50 $\pm$ 0.1**	5.9 $\pm$ 0.9*
GL/GS7.5	12.4 $\pm$ 2.3*	0.48 $\pm$ 0.09**	5.6 $\pm$ 1.1*
GL/GS10	13.2 $\pm$ 3.8*	0.62 $\pm$ 0.04**	4.4 $\pm$ 1.2**
GL/AgNPs2.5	8.1 $\pm$ 1.2	0.32 $\pm$ 0.08	6.1 $\pm$ 0.9*
GL/AgNPs5	22.2 $\pm$ 2.1**	0.55 $\pm$ 0.09**	2.8 $\pm$ 1.1**

3.5 Antibacterial agent release quantification

The GS release from GL nanofibres was evaluated *in vitro* by quantifying the amount of GS in the collected medium after different time periods. Fig. 3A shows the release kinetics of GS from nanofibrous matrices after 1, 3, 6, 9 and 24 h of incubation in PBS using UV–visible spectroscopy. The results revealed that all electrospun samples released a quick burst of antibiotic in the first 3 hours, with magnitude increasing with drug loading content. For longer exposure times, minimal drug release was observed for all samples. A rapid release of antibacterial agents during the first 3 hours would kill most of the pathogens, whereas a slow and constant release over the following hours would avoid re-colonization during the healing process.

The Ag⁺ ion release was measured through ICP-AES analysis which revealed a burst release of ions in the first hours as observed for GS (Fig. 3B). On the contrary, a sustained release was measured in the following days up to 7 days, confirming the long-lasting antibacterial effect of the GL/AgNPs nanofibers.

Fig. 3. (A) Cumulative release of GS from electrospun GL/GS2.5, GL/GS5, GL/GS7.5 and GL/GS10 blends. The GS release concentration was reported as a percentage respect to the initial concentration into the GL nanofibres. (B) Cumulative release of silver ions from electrospun GL/AgNPs2.5 e GL/AgNPs5.

3.6 Antibacterial activity of GL/GS and GL/AgNPs nanofibres

3.6.1 Disk diffusion method

The antibacterial activity of nanofibrous membranes against Gram-positive *S. aureus*, *S. epidermidis* and Gram-negative *P. aeruginosa* and *E. coli* was firstly tested by diffusion method. After 24 h of incubation at 37°C, composite nanofibres loaded with GS or AgNPs presented a clear zone of inhibition around the fibre mat and confirmed the antibacterial activity against all the bacteria (Fig. 4). GL nanofibrous membranes had no antibacterial activity against all the tested

strains whereas GL/GS2.5 was ineffective only against *P. aeruginosa*. GL/GS5, GL/GS7.5, GL/GS10 and GL/AgNO₃ (GL/AgNPs2.5 and GL/AgNPs5) nanofibrous membranes were effective both against Gram-positive and Gram-negative strains in a dose-concentration manner.

Fig.4 Images of the inhibition zones for GL based nanofibres: (A) drug-free GL, (B) GL/GS2.5, (C) GL/GS5, (D) GL/GS7.5, (E) GL/GS10, (F) drug-free GL, (G) GL/AgNPs2.5 and (H) GL/AgNPs5.

3.6.2 MTT colorimetric assay

The antibacterial activity of 2 mg of each sample was quantified by means of the MTT assay up to 72 hours. Fig. 5 shows the differences in metabolic activity of cells co-incubated for 24 hours

with nanofibrous membranes. In comparison to GL fibres, GL/GS and GL/AgNO₃ samples significantly reduced cell growth of all tested strains (p<0.001): In particular, *S. aureus* and *S. epidermidis* were inhibited averagely by 97%, *E. coli* by 98 % and *P. aeruginosa*, the less susceptible, by 93 %.

Fig.5. Metabolic activity measured by MTT assay of 4 different strains incubated for 24h with nanofibrous membranes loading different amount of GS (A) and Ag (B). Statistical analysis is referred to the comparison between the different nanofibres and the control at the same time point (*) p<0.001).**

After 48 and 72 hours of incubation, the antibacterial effect of GL/GS and GL/AgNO₃ nanofibrous membranes was confirmed by inhibition percentages > 98.5% for all the tested strains with the exception of *P. aeruginosa* co-incubated with GL/GS 2.5%, which was reduced by 95% (p<0.001) (reported as Supporting Information, Fig. S5).

3.7 Cellular test

NHDF-Neo were seeded onto the surface of the nanofibres membranes containing silver and gentamicin at different concentrations in order to evaluate the biological properties of these systems. Cell viability was evaluated by live/dead staining assay after 24 and 48 hours of incubation, as shown in Fig. 6. The bare GL membranes showed a high cell viability and ability to promote cell attachment. On the other hand, the presence of antibacterial agents affected the cell behavior when a threshold concentration was exceeded. Particularly, it was found that the addition of gentamicin to GL membranes (for both concentrations 2.5 and 10 %w/v) maintained the good cell viability observed on cells cultured on tissue culture plate TCP and GL samples. As shown in Fig. 6 (C-D) by the Live/dead and metabolic activity assays, cell viability was maintained and cell growth was enhanced after 48 hours. NHDF-Neo exhibited their typically flattened and elongated morphology on both membranes and spreading homogeneously along the sample surface. On the other hand, silver-loaded membranes, for both concentrations at 2.5 and 5 % w/v, evidenced lower cytocompatibility compared with GL and gentamicin-loaded samples.

The metabolic activity of NHDF-Neo was evaluated using Presto Blue assay (Fig. 7) up to 4 days. NHDF-Neo metabolic activity revealed a significant increase at each time point by incorporating gentamicin within the GL membranes in both concentrations, confirming the results observed by the live/dead staining assay. Interestingly, the GL/GS10 induced the highest fibroblasts metabolic activity when compared to GL and GL/GS2.5 ($p < 0.001$). The GS-loaded samples stimulated the cell growth and promoted a faster fibroblasts spreading.

In contrast to GS, a significant viability reduction was observed on nanofibrous membranes loaded with silver, as previously described for Live/Dead analysis. After 24 hours, a reduction of 50% compared to control was observed, however cells were able to proliferate after this initial cytotoxicity and to achieve a proliferation rate comparable to control after 4 days [59, 60]. In terms

of cell morphology, cell maintained spindle-shape and grow attached to GL substrates in the presence of GS in the presence of low concentrations of AgNPs, while cells at higher concentrations of AgNPs evidenced a rounded shape and cellular contraction with smaller nucleus (Fig. 8). This can be ascribed to a cytotoxic effect of AgNPs confirmed by Live/Dead staining and low metabolic activity detected by Presto Blue assay.

Fig.6 Live/Dead assay of cells seeded on membrane surfaces. Representative fluorescent micrographs of live (green) and dead (red) cells seeded on GL based nanofibres after 24 and 48 hours: (A) TCP, (B) drug-free GL, (C) GL/GS2.5, (D) GL/GS10, (E) GL/AgNPs2.5 and (F) GL/AgNPs5. Scale bars= 500 μ m.

Fig.7 PrestoBlue assay of cells cultured on based nanofibres after 1 and 4 days. Statistical analysis is referred to TCP at the same time point (* $p<0.05$, ** $p<0.01$, * $p<0.001$).**

Fig.8 Fluorescence microscopy of cells seeded on membrane surfaces. Representative fluorescent micrographs of cell morphology (nucleus stained with DAPI in blue, and actin filaments stained by Rhodamine in red) on GL based nanofibres after 48 hours: (A) drug-free GL, (B) GL/GS2.5, (C) GL/GS10, (D) GL/AgNPs2.5 and (E) GL/AgNPs5. Scale bars= 50 μm .

Discussion

An appropriate and ideal wound dressing should allow to (1) reduce infection and pain, (2) enhance the restoration of epithelial tissue, (3) be biocompatible, (4) prevent excessive fluid loss, and (5) preserve a moist environment for healing. Among others, electrospun matrices offer the unique

opportunity to combine a biomimetic, ECM-like and highly permeable substrate able to enhance tissue regeneration, with antibacterial agents to contrast bacteria adhesion and proliferation and consequently, to avoid the risk of infection during the wound healing process[61]. The possibility to fabricate ECM-like substrates is grounded in the design of the fabrication protocol which should reduce (and ideally avoid) the damage of the natural material structure in order to not compromise the interaction with cells. In this work, electrospun gelatin nanofibres with antibacterial properties were produced using an environmental and cell friendly method where only water was used as solvent within all the process to reduce the risk of cytotoxicity due to non-physiological residues and to avoid gelatin denaturation during the fabrication process [62]. The pivotal role of scaffold composition in regulating cell adhesion and proliferation has been reported since decades [63] and ECM-derived proteins are widely used in tissue engineering approaches [64]. GL was selected due to its solubility properties, as it can be solubilized in water without the use of acidic conditions or solvents which could alter the antibacterial efficacy of the antibacterial agents and could strongly affect the biocompatibility if any residue remains entrapped into the final devices. The possibility to work without the use of aggressive and potentially cytotoxic solvents is a relevant topic in the current trends for scaffolds fabrication. Compared to the state-of-the-art [65], the novelty of this work lies in the combination of nanofibrous matrices (mimicking the morphology of the extracellular matrix) with a biomimetic polymer (gelatin) using a “green electrospinning” method avoiding the use of environmental and cellular unfriendly solvents.

The nanometer-sized fibrous morphology was selected to provide a high surface area to contact with cells and bacteria resulting in both good tissue regeneration and enhanced antibacterial effectiveness. The nanofibrous structure was obtained for all the fabricated samples (Fig.1 and Fig.2) and the presence of antibacterial agents slightly modified the fibres size and distribution.

Both GS and AgNP were loaded within the GL-nanofibres as confirmed by EDS, TEM and FTIR analysis (Fig.1, Fig.2 and Fig.S3). Furthermore, GS and AgNP doped nanofibres showed anhydrophilicity within the range 50°-70° (Table 1). The hydrophilicity of biomaterials has a great influence on the adhesion and proliferation of cells and the effect of doping agents on nanofibres hydrophilicity was measured to confirm that the changes in hydrophilicity are within the optimal range for cell adhesion [54]. Moreover, for wound dressings, the higher hydrophilic properties provide several advantages such as the maintenance of moist environments, exacerbated exudates or blood adsorption, and also high water vapor permeability at the wound site [55]. Besides hydrophilicity, mechanical properties were influenced by the presence of the antibacterial agents. A reinforcement of the nanofibers was observed when 10% GS and 2.5% and 5% AgNPs were added (Table 2 ND Fig. S4).

Both GS and Ag were released after soaking into aqueous environment. A burst release was observed within the first three hours (Fig.3) for both the antibacterial agents. Then, the GS reached a maximum within 24 hours while Ag⁺ was released up to 7 days confirming a more prolonged antibacterial effect for AgNPs doped nanofibers. As reported in literature, GS is one of the most important antibiotics used for different applications, mainly for the treatment of osteomyelitis and wound healing [57]. In order to achieve the effect of antibiosis, the local concentration of gentamicin should be released constantly and above 32 mg/mL [58]. Therefore, a local delivery system of gentamicin should be designed to maintain the effective concentration to treat wounds as reported in this *in vitro* work.

The release of these agents conferred antibacterial activity against bacterial strains usually found in infected wounds namely *S. aureus*, *S. epidermidis*, *P. aeruginosa* and *E. coli* (Fig. 4, Fig. 5 and Fig. S5). Few systems based on electrospun membranes have been reported to offer a unique

combination of the inherent properties, such as promoting cell proliferation, and simultaneously provide anesthetic and antibiotic activity for pain relief and healing [66]. However the antibiotics content should be evaluated carefully, because it may tackle the epithelial regeneration [67]. Considering this challenge, *in vitro* tests were performed using NHDF-Neo to analyse the effect of each antibacterial agent on cell viability and morphology. Gelatin-based substrate has been reported to favour cellular adhesion, due to the presence of cell-binding motifs (i.e. RGD) in gelatin that mediate cell-substrate interactions [68] as well as the presence of interconnected pores and the rough surface created by the nanofibrous membrane structure that allows to support the proliferation and quicker regeneration of cells [8]. However, the addition of the antibacterial drugs could affect the cellular behavior as cytotoxic effect has been reported for certain amounts of the antibacterial agents [69, 70]. In our work, GS loading amount does not affect the viability and morphology of neonatal normal human dermal fibroblasts in the presence of all the concentrations tested. However a reduction of cell viability was observed for Ag loading samples after 24 hours. These findings were supported by the ICP analysis which revealed an initial burst release of Ag⁺ which could overcome the biocompatibility threshold of silver. It is generally accepted assumption that the bactericidal amount of silver may have toxic effects to the cells (mainly towards prokaryotes than mammalian cells) [59, 60]. Although a slight cytotoxicity after 24h was observed, results at 4 days showed an increase in cell number and a proliferation rate comparable to the control confirming the ability of the nanofibrous matrices to support proliferation even if an initial viability reduction occurred probably ascribed to a reduction of cell adhesion when Ag was loaded into the nanofibrous mats. Interestingly, NHDF-Neo cell maintained and spindle-shape when cultured on GL substrates in the presence of GS and low concentrations of AgNPs confirming the effect of the nanostructured topography on cell arrangement.

Conclusion

Electrospun gelatin nanofibres with antibacterial properties were produced to avoid the risk of infection during the wound healing process. Reported data confirmed the ECM-like morphology of the nanostructured matrices which is a key aspect for cell adhesion and tissue regeneration. Furthermore, the antibacterial agents loaded into the nanofibrous mats are efficient against Gram-positive *S. aureus*, *S. epidermidis* and Gram-negative *P. aeruginosa*, *E. coli* bacteria. In conclusion, the developed matrices are promising membranes for application in wound healing thanks to the dual features combining support to tissue growth and antibacterial properties. Future work will be addressed to analyze the versatility to the proposed approach combining different antibacterial agents to achieve a multi-strains antibacterial effect. Furthermore, biomolecules (e.g. growth factors) able to enhance the regeneration process will be loaded into the developed nanofibers with limited risks of biomolecules denaturation thanks to the “green electrospinning approach” based on highly biocompatible process conditions.

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Authors declare no conflict of interests.

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Table of Contents/Abstract Graphic

Novel scaffolds with biomimetic features and antibacterial properties for tissue regeneration after skin injuries.

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