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Glycosaminoglycan functionalization of electrospun scaffolds enhances Schwann cell activity

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ABSTRACT

Nerve fibers of the peripheral nervous system (PNS) have a remarkable ability to regenerate up to an almost complete recovery of normal function following a crush or a Sunderland Type II injury. This process is governed by glial cells, known as Schwann cells, through their unique capacity to dedifferentiate into cells that drive the healing process. Despite that many progresses have occurred in restorative medicine and microsurgery, the regenerative process after a severe lesion of a major nerve trunk (e.g., Sunderland Types III–V) is often incomplete and functional recovery is unsatisfactory. In this aspect, it is known that glycosaminoglycans (GAGs) of the extracellular matrix are involved in proliferation, synaptogenesis, neural plasticity, and regeneration of the PNS. Here, we developed poly(caprolactone) (PCL) fibrous scaffolds functionalized with GAGs, which allowed us to assess their influence on the adhesion, proliferation, and differentiation of Schwann cells. We found that both aligned and random fiber scaffolds functionalized with GAGs resulted in increased cell proliferation on day 1. In addition, aligned functionalized scaffolds also resulted in increased GAG presence on day 1, probably because of cell extracellular matrix (ECM) formation and an increased syndecan-4 expression on day 7. A different modification and activation of Schwann cells in the presence of GAG versus no-GAG scaffolds was underlined by proteomic comparative analysis, where a general downregulation of the expression of intracellular/structural and synthetic proteins was shown on day 7 for GAG-functionalized scaffolds with regard to the nonfunctionalized ones. In conclusion, we have shown that GAG-functionalized scaffolds are effective in modulating Schwann cell behavior in terms of adhesion, proliferation, and differentiation and should be considered in strategies to improve PNS repair.

Statement of Significance

Nerve fibers functional recovery following a severe trauma of the Peripheral Nervous System (PNS) still represents a huge challenge for neurosurgery nowadays. In this respect, tissue engineering is committed to develop new constructs able to guide Schwann cells by mimicking the natural extracellular matrix environment. To this purpose, we successfully fabricated polycaprolactone (PCL) scaffolds with two well-defined fiber deposition patterns, functionalized with glycosaminoglycans (GAGs) and assessed for their potential as support for Schwann cells adhesion, growth and differentiation, by both classical biochemistry and LC-MS-based proteomic profiling. By this way, we showed that PCL–GAGs scaffolds could represent a promising artificial substrate that closely mimics the recently established pattern of Schwann cells migration into the regenerating nerve and, therefore, it should be considered in strategies to improve PNS repair.

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1. Introduction

Nerve recovery from peripheral nerve trauma depends on different factors including the extent of the lesion, time course, and recovery following nerve injury. When a peripheral nerve undergoes a traumatic injury, several pathophysiological processes occur at the proximal and distal segments of the injury site, as well as in the nerve cell body and the distal endings of both muscle end plates and sensory receptors. Changes in the nerve at the site of injury begin almost immediately, and the healing process is guided by the glial cells of the peripheral nervous system (PNS), called Schwann cells, which are critical in the regeneration of a mature nerve [1]. However, when the gap between proximal and distal segments is too large, complete self-regeneration is not possible and microsurgical intervention is essential for nerve repair [2–4]. The current “golden standard” to treat neural defects employs autologous grafts [5–7]. However, autografts have limitations, which include shortage of nerves, as they are taken from the same patient, a mismatch between the sizes of the donor site and the recipient site, neuroma formation, and lack of functional recovery [8,9]. An alternative to autografts is the use of allogenic grafts isolated from cadavers. These grafts, however, may undergo host-graft immune rejection [10]. To overcome this limitation, several studies have been conducted to evaluate the efficiency of acellular nerve grafts [11,12].

In the last few years, some disciplines such as nanotechnology [13,14] and tissue engineering [15–18] have focused on this issue. The major challenge in tissue engineering is to develop a synthetic support structure, or scaffold, that is able to mimic the natural extracellular matrix (ECM). A synthetic ECM should have several features including biocompatibility, controlled biodegradability with nontoxic products, low inflammatory potential, porosity promoting cell attachment and growth, and mechanical properties similar to those of the tissue to be replaced [19–21]. In these aspects, polymeric biomaterials are widely used for scaffold fabrication. Presently, there is a vast choice of both synthetic polymers and natural materials displaying the required properties mentioned above [22], but few clinically approved nerve conduits are available [23–25]. Several techniques have been used to fabricate tissue engineering scaffolds such as electrospinning, nanofiber self-assembly, liquid–liquid phase separation, solvent casting and particulate leaching, emulsification/freezing-drying, gas foaming, and additive manufacturing [26–29].

Biocompatibility of engineered scaffolds could be further enhanced by surface modification of the material, in particular, by attaching bioactive molecules. Glycosaminoglycans (GAGs) are a family of highly charged, unbranched heteropolysaccharides that play key roles as structural and functional components of the extracellular matrix. Except for hyaluronic acid (HA), they are variably sulfated and covalently linked to protein cores, thereby forming proteoglycans (PGs). GAGs are structurally very heterogeneous in terms of both type and number of repeating disaccharide building blocks, degree and pattern of sulfation, and degree of hexuronic acid epimerization. Numerous biological functions of PGs, such as cell interactions, differentiation, proliferation, and matrix assembly, mainly depend on specific structural characteristics of their GAG chains in both physiological and pathological conditions [30,31].

In the central nervous system (CNS), sulfated proteoglycans such as chondroitin sulfate proteoglycans (CSPGs), dermatan sulfate proteoglycans (DSPGs), keratan sulfate proteoglycans (KSPGs), and heparan sulfate proteoglycans (HSPGs) represent major functional constituents of the extracellular matrix [32–34]. They have a role in several physiological processes. For example, during embryonic development, they stimulate processes such as

neuronal adhesion, migration, and neurite formation [35–38]. Additionally, they are involved in the restoration of neuronal function following a trauma, and their effects strictly depend on structural characteristics of GAGs. In particular, the hyaluronan CSPG family inhibits neurite outgrowth, whereas other PGs containing disulfated CS disaccharide units (4-O-sulfate or 6-O-sulfate) or oversulfated DS disaccharide units are neurostimulatory and promote repair processes [34].

Interestingly, in the PNS, it has been found that, after a peripheral nerve injury, chondroitin 6-sulfotransferase-1 (C6ST-1) is upregulated, suggesting that an increase in 6-sulfated GAGs makes the ECM more prone to axonal regeneration, and the balance of different CS GAGs in the microenvironment around the lesion is an important factor in determining the response of the nervous system after injury [39,40]. On the other hand, it has been reported that chondroitinase treatment may promote axonal regeneration in both CNS and spinal cord tissues [41,42]. Porous PDLLA scaffolds [43], hydrogels [44–46], and carbon nanotubes [47] functionalized with CS have been fabricated, representing promising constructs for neuronal regeneration.

The aim of this study was to evaluate the response of Schwann cells grown on electrospun scaffolds functionalized with specific GAGs purified from the aorta porcine tissue. In this aspect, understanding whether GAGs can improve proliferative activities and binding capacities of the cells to the scaffolds could be promising to improve the process of nerve regeneration. Proliferation, metabolic activity, and GAG assays were performed. The expression of specific markers (i.e., Syndecan 1, Syndecan 4, Integrin $\alpha 9 \beta 1$, Laminin, and p75) during 7 days of culture was evaluated by both immunofluorescence and western blot analyses. Furthermore, a proteomic approach was applied for the evaluation of eventual specific patterns of protein expression after 3 days and 7 days of culture.

2. Materials and methods

2.1. Preparation of electrospun scaffolds

Electrospinning produces a sheet of fibers by pushing a liquid jet through a nozzle under an electric field [48–53]. Electrospun polycaprolactone (PCL, 45 kDa, Sigma-Aldrich, Germany) fiber mats used as fibrous scaffolds were fabricated by electrospinning from a 15% (w/v) PCL solution in 1:4 (v/v) dimethylformamide (DMF): chloroform. A blunt 20-gauge stainless steel hypodermic needle (o.d. 0.8 mm, Unimed, Switzerland) was used as the nozzle. A polyurethane nonwoven mesh (a kind gift from Lantor B.V., The Netherlands) and an aluminum foil wrapped around a rotating cylinder (cylinder size = o.d. 6 cm, length 19 cm) were used as the collector. The working distance from the tip of the needle to the surface of the aluminum sheet was 14 cm. A high-voltage power supply (Gamma High Voltage Research Inc., FL, USA) was used to generate a direct current potential of 20 kV. The emitting electrode of positive polarity was connected to the needle, while the grounding one was connected to the collector. The feed rate of the PCL solution was controlled using a syringe pump (KDS 100, KD Scientific) at 1 mL/h. A 60 mm diameter mandrel speed was rotated at 500 rpm (2.6 V) to obtain scaffolds with random fibers and approximately 4000 rpm (9 V) to obtain scaffolds with aligned fibers. The needle was set to move back and forth along the length of the cylinder to permit homogeneous fiber deposition on top of the polyurethane sheet. After continuous spinning for 15 min, homogenous PCL fibrous scaffolds were obtained. The morphology of the fibrous scaffolds and the size of the individual fiber segment therein were examined using a Philips XL30 scanning electron microscope. Multiple scanning electron microscopy

(SEM) images in quadruplicate for each condition were statistically analyzed using Fiji image processing package (GNU General Public License), by which the mean value of the diameters of the fiber segments within the PCL fibrous scaffolds was determined (five different fibers in distinct points of each image in quadruplicate for each condition, resulting in at least 20 fibers per scaffold group).

2.2. Glycosaminoglycans purification

GAGs were purified as described by Naso et al. [54] and Cigliano et al. [55]. In brief, porcine aortic root from 10- to 12-month-old pigs were obtained from a local slaughterhouse. Fatty adherences were removed from aortic tissue by gentle peeling. The wet weight of each sample was determined after gently blotting with a filter paper. Aortic minced tissues were dehydrated with 20 volumes of acetone at 4 °C for 24 h, defatted with 20 volumes of chloroform:methanol (2:1, v/v) at 4 °C for 24 h, dried for 24 h at 60 °C after centrifugation at 3300×g for 15 min, and finally weighed (dry-defatted tissue (DDT) weight). DDTs were rehydrated for 24 h at 4 °C in 0.1 M sodium acetate buffer, pH 5.8, containing 5 mM cysteine, and 5 mM ethylenediaminetetraacetic acid (EDTA) (37 volumes per gram of DDT). Then, papain (0.3 U/mg of DDT) was added to the mixture, which was incubated at 56 °C for 48 h. The digestion was stopped by boiling the solution at 100 °C for 5 min, followed by centrifugation (9000×g for 15 min at 4 °C). The supernatant was loaded on a diethylaminoethyl (DEAE) Sephacel column (1.5 × 50 cm, 70 mL), equilibrated with a 50 mM sodium acetate buffer, pH 6.0. The column was then exhaustively washed with the same buffer (absorbance of flow-through fraction at 280 nm <0.05) and GAGs eluted with a 50 mM sodium acetate buffer, pH 6.0, containing 1.5 M NaCl. Fractions of 5 mL were collected and assayed for hexuronic acid (UA) content by the method of Bitter and Muir, using glucuronolactone as a standard [56]. GAG-containing fractions were pooled and precipitated using 4 volumes of cold absolute ethanol. The mixture was left overnight at –20 °C, and the precipitate was separated by centrifugation, washed twice with ethanol, and then dried. GAG composition was determined by discontinuous cellulose acetate electrophoresis [57]. Alcian blue-stained strips were acquired (Gel Doc XR System) and analyzed using Quantity One 4.6.3 software (Bio-Rad Laboratories).

Fine structural characterization of CS isomers was performed as previously described [58]. Briefly, following chondroitin ABC lyase treatment of CS isomers, unsaturated disaccharide units were fluorescently tagged with 2-aminoacridone and resolved by fluorophore-assisted carbohydrate electrophoresis (FACE). Images were acquired by UV transillumination (Gel Doc XR System) and analyzed by using Quantity One 4.6.3 software (Bio-Rad Laboratories).

2.3. Electrospun scaffold functionalization

PCL scaffolds were first aminolysed and then functionalized with GAGs (Supplementary Information, Fig. S1). First, dried scaffolds were incubated for 1 h at 37 °C with 5% 1,6-hexamethylenediamine (HMDA) in isopropanol and then washed three times with water. GAGs purified from porcine aorta tissue (100 µg UA solubilized in 100 µL H₂O) were added to each scaffold. As control, nonfunctionalized (nude) scaffolds were used; in this case, 100 µL of water was added. A solution of 50 mM N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC)/N-hydroxysuccinimide (NHS) in 2-(N-morpholino)ethanesulfonic acid (MES) was added in each scaffold and left overnight at room temperature. Before seeding, nonfunctionalized and functionalized scaffolds (o.d. 15 mm) were placed into a 24-well nontreated tissue culture plate (VWR). Each scaffold was pushed to the bottom of the well with O-rings (Eriks BV, The Netherlands) to prevent cells from

migrating out of the scaffolds onto the culture plate. Afterwards, supplemented (L-glutamine, FBS, penicillin-streptomycin) DMEM was added in each well, and the plate was incubated at 37 °C with 5.0% CO₂ for 24 h. To assess the degree of GAG functionalization, an Alcian blue staining protocol was developed. In brief, scaffolds were placed in a 24-well-plate and stained with Alcian blue solution (0.1% Alcian blue, 10% EtOH, 0.1% CH₃COOH, 0.03 M MgCl₂ in water) for 30 min at room temperature. Then, the scaffolds were washed with Alcian blue destaining solution (10% EtOH, 0.1% CH₃COOH, 0.03 M MgCl₂ in water) for 30 min at room temperature. To dissolve Alcian blue bound to the GAGs linked to the scaffolds, a solution of 1% SDS (w/v) was added into each well, and the plate was left shaking (200 rpm) for 30 min. The supernatant was transferred into a new well plate, and absorbance at 605 nm was read with the CLARIOstar plate reader (BMG Labtech).

2.4. Cell culture studies

Four types of constructs were produced for cell culture studies: scaffolds with random fibers (R), random fibers functionalized with GAGs (RG), aligned fibers (A), and aligned fibers functionalized with GAGs (AG).

Neuronal Schwann cells RT4-D6P2T (ATCC® CRL-2768TM) were maintained in DMEM high glucose (Thermo Fisher Scientific) supplemented with 2 mM L-glutamine (Thermo Fisher Scientific), 10% fetal bovine serum (FBS; Sigma), and penicillin–streptomycin (100 U/mL; Sigma). Cells were removed from the culture flasks using a trypsin-EDTA solution (Sigma) after reaching confluence. Before seeding, the viable cell number was determined by live cell staining using 0.4% trypan blue (w/v) (Sigma), followed by counting with Neubauer chamber. A Schwann cell suspension was pipetted onto the surface of the dry scaffolds at a density of 2×10^4 cells/well. Wells were then filled with culture medium to reach the final volume of 500 µL; the plate was then placed into a cell culture incubator, and cells were maintained at 37 °C with 5.0% CO₂ for different time points (1, 3, and 7 days). We chose to perform the experiments with a cell line because primary SCs rapidly stop their metabolic activity in the absence of axons and begin to downregulate many myelin constituents, thus hindering cell biology studies. Several studies have shown that the rat Schwann cells cell line RT4-D6P2T is a practical alternative and an appropriate model for these kinds of studies [59,60].

2.4.1. Cell metabolic studies

To measure the metabolic activity of Schwann cells seeded in scaffolds, PrestoBlue® Cell Viability Reagent (Thermo Fisher Scientific) was used. At the first time point (day 1), 50 µL of 9% PrestoBlue (w/v) in DMEM was added to each well as an indicator of viable cells. After incubation at 37 °C for 30 min, the solution of PrestoBlue/DMEM was transferred into a new well plate, and fluorescence between 535 and 590 nm was assessed using a CLARIOstar plate reader. Then, cells were rinsed with PBS, and 500 µL of DMEM was added to each well. Scaffolds with cells were incubated at 37 °C, and the metabolic assay was repeated on days 3 and 7.

2.4.2. Dna assay

To determine the seeding efficiency and cell growth on the scaffolds, DNA was quantified by a fluorometric DNA assay on cell lysates. At each time point, the medium was removed and scaffolds with cells were rinsed with PBS, dried, and transferred to a new Eppendorf tube. Each scaffold was frozen at –80 °C for at least 24 h. Then, samples were thawed, and the DNA content was measured using a CyQuant cell proliferation assay kit (Invitrogen) following the manufacturer's instructions. Samples were incubated with 250 µL of Proteinase K (VWR) digestion buffer (1 mg/mL Proteinase K in Tris-EDTA digestion buffer) at 56 °C for 16 h. Forty

microliters of the sample was transferred to a black 96-well plate and treated with 40 μ L/well RNase for 1 h at room temperature to assure DNA specificity. Then, 80 μ L of CyQuant reagent dye was added, the samples were incubated for 15 min in the dark at room temperature, and fluorescence signals (excitation 485 nm, emission 520 nm) were detected using the CLARIOstar plate reader. DNA quantification was performed using a standard curve ranging from 0.5 μ g/mL to 2 μ g/mL of λ DNA, provided by the manufacturer. The number of cells was calculated assuming that approximately 6.6 pg of DNA is present in each mammalian cell as also described in literature [61].

2.4.3. Neosynthesized gags quantitation

To quantify GAGs produced by cells seeded on scaffolds during culture, spectrophotometric assays based on 1,9-dimethylmethylene blue (DMMB) solution (38 mM DMMB, 9.5 mM HCl, 40.5 mM glycine, 40.5 mM NaCl in water, pH 3) were performed. The DMMB solution was stable for at least 4 months when stored at room temperature in the dark. To extract GAGs from cells at 1-, 3-, and 7-day time points, the medium was removed, and scaffolds with cells were rinsed with PBS and frozen at -20°C for at least 24 h. Then, samples were incubated with 250 μ L of Proteinase K (VWR) digestion buffer (1 mg/mL Proteinase K in Tris-EDTA digestion buffer) at 56°C for 16 h. Twenty-five microliters of each sample was transferred into a black 96-well plate. Five microliters of 2.3 M NaCl and filtered DMMB solution (150 μ L) was added into each well. The absorbance was directly measured at 525 nm using the CLARIOstar plate reader. A standard curve ranging from 1 μ g/mL to 10 μ g/mL was generated by using a standard GAG solution (chondroitin 4-sulfate) dissolved in Proteinase K digestion buffer with 10 μ g cysteine in 1 mL PBE (0.2%).

2.4.4. Immunostaining and fluorescence microscopy analyses

Schwann cells were fixed for 20 min with 4% (w/v) paraformaldehyde at 4°C and rinsed several times with PBS. Cells were permeabilized with 0.1% (v/v) Triton X-100 (VWR) in PBS for 15 min at room temperature and then incubated in blocking solution containing 1% (w/v) bovine serum albumin (BSA, VWR), 5% (v/v) goat serum (Sigma), and 0.05% (v/v) Tween-20 (VWR) in PBS for 1 h. Afterwards, cells were incubated with primary antibody overnight at 4°C . The primary antibodies used were anti-laminin $\beta 2\gamma 1$ rabbit antibody (1:250, BioConnect), anti-integrin $\alpha 9\beta 1$ mouse antibody (1:1000, Abcam), anti-syndecan 1 mouse antibody (1:1000, Abcam), and anti-syndecan 4 rabbit antibody (1:500, BioConnect). Cells were extensively washed with 1% BSA and 0.05% Tween 20 in PBS and incubated with secondary antibody for 1 h at room temperature. Alexa Fluor-488 goat antimouse and Alexa Fluor-647 goat antirabbit (both 1:500, Invitrogen) were used as secondary antibodies. F-actin was visualized by incubating cells with Phalloidin Alexa Fluor 568 (5 μ L/205 μ L in washing buffer, Thermo Fisher Scientific). Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (for 5 min at 0.1 μ g/mL; Sigma). Cells were extensively washed with PBS and mounted in Mowiol 4–88 (Sigma) at 4°C until imaging. Samples were examined by fluorescence microscopy, and images were obtained with a Nikon Eclipse Ti2 inverted microscope. Image adjustments were limited to contrast enhancement and level settings using Nikon NIS Elements software and Fiji image processing package (GNU General Public License).

2.4.5. Western blotting analyses

At each time point, cells were placed on ice, washed with ice-cold PBS, and subjected to lysate treatment. Lysis buffer included complete protease inhibitors (Sigma), 2 mM phenylmethylsulfonyl fluoride (PMSF, Sigma), and 84.7% v/v RIPA buffer (Sigma). To minimize the presence of cell debris, protein lysates were centrifuged

(10000 $\times$ g) for 10 min at 4°C , and the supernatant was collected and stored at -80°C . To detect Syndecan 4 neoepitopes, protein lysates were pretreated with Heparinase II (2.5 mU/ μ g protein, Sigma) overnight at 37°C . Protein samples were quantified with Pierce BCA protein assay kit (Thermo Fisher Scientific) and treated with an equal amount of Laemmli Sample buffer (Bio-Rad) added with 10% 2-mercaptoethanol (Sigma) at 100°C for 5 min. For immunodetection, 10 μ g of proteins from each sample was loaded, and the proteins were separated in 4–15% Mini-PROTEAN TGX Stain-Free Gel (Bio-Rad) and transferred by electroblotting to PVDF membranes (Bio-Rad). Membranes were blocked with 2.6% Blocking-Grade buffer (Bio-Rad) in TBST (0.1% Tween 20 in TBS) and incubated overnight at 4°C in the same solution with anti-laminin $\beta 2\gamma 1$ rabbit antibody (1:250, BioConnect), anti-integrin $\alpha 9\beta 1$ mouse antibody (1:500, Abcam), anti-syndecan 1 mouse antibody (1:250, Abcam), anti-syndecan 4 rabbit antibody (1:250, BioConnect), anti-p75 NGF receptor rabbit antibody (1:500 Abcam), and anti- β -actin mouse antibody (1:2500, Sigma). Goat antirabbit IgG and goat antimouse IgG, conjugated with horseradish peroxidase from Bio-Rad (1:1500 dilution), were used as secondary antibodies. Following incubation with chemiluminescence western blot substrate (Clarity Western ECL Blotting Substrate, Bio-Rad), Grayscale images were acquired using ChemiDoc XRS System (Bio-Rad) and analyzed by using Fiji software (GNU General Public License). Band intensities of each marker were normalized using β -actin as the housekeeping protein.

2.4.6. Proteomic analyses

NanoLC-MS/MS SWATHTM analyses were performed on Schwann cell lysates from R, RG, A, and AG scaffolds after 7 days of culture. Three replicates of each culture condition were produced for proteomic analyses. Schwann cells on scaffolds were directly lysed in lysis buffer (50 mM Tris HCl, 1% Triton X100, 0.25% Sodium dodecyl cholate, Protease Inhibitor), vortexed for 30 min, frozen for 10 min, and finally sonicated for 15 min. After centrifugation at 16,000 $\times$ g for 10 min, the supernatant was collected, and protein concentration was measured through BCA assay. The detergent was removed through a column (Detergent removal column Pierce), and 50 μ g of protein was processed by adding 10% acetonitrile (ACN, Romil, UK) in 50 mM ammonium bicarbonate, and 5 mM dithiotreitol at 80°C for 20 min, followed by alkylation with 10 mM iodoacetamide in the dark at 37°C . Digestion was performed by incubating the substrate with trypsin (Roche, Germany) (enzyme:protein ratio, 1:100) overnight at 37°C . Samples were acidified and then loaded on a C18 cartridge to purify peptides; solutions were evaporated in few minutes in SpeedVac (Savant) and eventually diluted to a final volume of 100 μ L with 2% ACN/0.1% formic acid (FA); 3 μ L of the sample was injected for library generation and a duplicate of 2 μ L of each sample for SWATHTM method analysis. Chromatographic separation of peptides was performed using a nano-HPLC system (Eksigent, AB Sciex, USA). Samples were loaded in a precolumn cartridge (PepMap-100 C18 5 μ m 100 A, 0.1×20 mm, Thermo Fisher Scientific, USA) and then separated in a C18 PepMap-100 column (3 μ m, 75 μ m $\times$ 250 mm, Thermo Fisher Scientific, USA) at a flow rate of 300 nL min⁻¹. Runs were performed with eluent A (ultrapure water, 0.1% FA) under 60 min of linear gradient from 5% to 40% of eluent B (ACN/0.1% FA), followed by 10 min of a purge step and 20 min of the re-equilibration step. Peptides eluted from chromatography were directly processed using a TripleTOFTM 5600-ESI mass spectrometer (AB Sciex, USA). Data were acquired using the SWATHTM method for shotgun data-independent MRM quantification. Library was generated by processing MS/MS data through ProteinPilotTM Software (AB Sciex, USA).

Differentially expressed proteins were mapped onto STRING v10 to build functional protein association networks with

reliability score higher than 0.4. STRING v10 is a database of known and predicted physical/functional protein associations based on four sources: genomic context, high-throughput experiments, co-expression, and previous knowledge of functional protein association networks.

2.5. Statistical analysis

Each experiment was carried out in triplicates, except for western blot (quadruplicates). All results are presented as mean $\pm$ standard deviation (SD). GraphPad Prism software v 4.03 (GraphPad, San Diego, CA, USA) was used to perform statistical analysis. Statistical significance was set at a p -value ≤ 0.05 .

For proteomics data obtained by mass spectrometry, the false discovery rate (FDR) analysis was set to a confidence level of 95%. The comparative analysis was performed using PeakView™ Software (ABSciex, USA) with MS/MS (ALL) in SWATH™ Acquisition MicroApp 2.0 and MarkerView™ (AB Sciex, USA). Retention time alignment was obtained using selected peptides (top confidence and top-level transitions) from top score protein. Processing settings were 7 peptides per protein, 7 transitions per peptide, 93% peptide confidence (according to Paragon algorithm result), and 5% FDR; XIC options: extraction window 10 min, width 50 ppm, and 0.1 Da. Normalization was done using a global normalization

of total ion profiles (total protein content). Principal Component Analysis (PCA) was performed to evidence groupings among the data set. Groups were compared with t -test using a threshold of 95% (p value = 0.05) and fold change > 2 .

3. Results

3.1. Electrospun scaffold fabrication

To determine how the rotation of a grounded mandrel affects the scaffold structure, the mandrel speed was set at 500 and 4000 rpm. Under baseline conditions (mandrel rpm = 500) PCL solution produced scaffolds composed of $1.34 (\pm 0.23) \mu\text{m}$ diameter fibers. At 4000 rpm mandrel speed, scaffolds were made up of $1.35 (\pm 0.04) \mu\text{m}$ diameter fibers. In both cases, fibers had a diameter similar to that of collagen bundles [62]. Fibers produced with different mandrel speeds had a rounded cross-sectional profile, and the mandrel rotation did not alter the average fiber diameter. The fiber alignment was crucial to mimic the oriented pattern of collagen fibrils found in the ECM during nerve regeneration [62,63]. In this aspect, scaffold images obtained with SEM showed that fibers obtained with a mandrel speed of 4000 rpm were aligned compared to the fibers in scaffolds produced at 500 rpm mandrel speed. As illustrated in Fig. 1, analysis revealed that the

Fig. 1. Dispersion analysis of the two typologies of fabricated PCL scaffolds. Representative SEM images of random (mandrel speed = 500 rpm) and aligned (mandrel speed = 4000 rpm) fibers (panels A and B, respectively) and the corresponding directionality histograms (panels C and D) obtained by using Fiji software. Histogram showing dispersion average and standard deviation in both random and aligned fibers (panel E).

dispersion of fibers was much narrower for scaffolds with aligned fibers than for scaffolds with random fibers, indicating that aligned fibers achieved a higher degree of orientation.

3.2. Gag analysis

GAGs were purified from porcine vascular tissue. Quantitative analysis evidenced GAG levels corresponding to $3.50 (\pm 0.08) \mu\text{g UA/mg dry weight}$ and $1.17 (\pm 0.03) \mu\text{g UA/mg fresh weight}$.

Fig. 2. Scaffold sterilization. From left to right: scaffolds sterilized with EtOH before functionalization with GAGs, scaffolds functionalized without the sterilization step, and scaffolds sterilized after crosslinking reaction. The amount of GAGs used to functionalize scaffolds was $100 \mu\text{g}$. ** $p < 0.01$.

Qualitative analysis showed the following GAG distribution in aortic wall in accordance with data previously reported by Naso et al. [54]: 60% chondroitin sulfate, 20% dermatan Sulfate, 13% heparan sulfate, and 7% hyaluronan (Supplementary Information, Fig. S2). Fine structural characterization of CS isomers showed that they were composed of 3% 4,6S disaccharide units, 47.9% 4S disaccharide units, 45.5% 6S disaccharide units, and 3.6% non-S disaccharide units, according to our previous study [55].

3.3. Scaffold functionalization

To evaluate the efficiency of PCL crosslinking with GAGs purified from aorta porcine tissue, an Alcian Blue staining protocol was developed. Compared to their corresponding controls, scaffolds pretreated with HMDA had a percentage of GAG coating higher than that in scaffolds hydrolyzed with NaOH (Supplementary Information Fig. S3).

Regarding scaffold sterilization, another experiment was set up to verify the efficiency of functionalization associated with a step of 2 h in 70% ethanol before or after the crosslinking reaction (Fig. 2). Functionalization was more efficient when scaffolds were not sterilized or when a sterilization step was preceded by the crosslinking reaction. Since aminolysis reaction occurs in isopropanol, we chose not to sterilize scaffolds, hence avoiding an additional step in the protocol. Indeed, scaffolds incubated with $100 \mu\text{g}$ of GAGs without EDC-NHS showed no differences with regard to scaffolds incubated with EDC-NHS without GAGs.

Fig. 3. Histograms showing metabolic and proliferation activities, as well as GAG and total protein production in Schwann cells during 7 days of 3D scaffold culture. (A) Cell metabolic activities assessed by Presto Blue assay. (B) Proliferation activity measured by DNA assay. (C) Biosynthesis of GAGs assessed by DMMB assay. (D) Protein biosynthesis assessed by BCA assay. Abbreviations: R = random, RG = random with GAGs, A = aligned, AG = aligned with GAGs. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Furthermore, Fig. S4 confirmed that GAGs did not specifically bind to the scaffold and validated the efficacy of EDC-NHS crosslinking in creating GAG-functionalized polymer nanofibers.

3.4. Cell studies

Results of metabolic activity (Fig. 3A) and proliferation (Fig. 3B), as well as GAGs and total protein production (Fig. 3C, D) by Schwann cells seeded in the four topologies of fabricated scaffolds, showed an overall increase in these activities during the 7 days of culture. The presence of GAGs reduced cell metabolic activity and GAG production in random scaffolds but did not have an effect on aligned fibers.

Immunofluorescence analysis clearly showed that Schwann cells seeded in scaffolds with aligned fibers had an elongated shape that followed the direction of the fibers. Conversely, cells seeded in scaffolds with random fibers assumed a spreaded morphology (Fig. 4). Furthermore, cells expressed laminin (Fig. 5), $\alpha 9\beta 1$ integrin (Fig. 6), syndecan 1 (Fig. 7), and syndecan 4 (Fig. 8) on the scaffolds. The same proteins, as well as p75, were quantified by western blot analysis (Fig. 9). $\alpha 9\beta 1$ Integrin is a widely distributed multifunctional receptor that interacts with a variety of endogenous ligands and ECM components. Furthermore, this isoform is a receptor for nerve growth factor (NGF) and two other neurotrophins, namely, brain-derived neurotrophic factor and NT3 [64]. $\alpha 9\beta 1$ Integrin showed decreased levels from days 1 to 3 and day 7 (Fig. 9A), although not statistically significant. This protein is known to be involved in focal adhesion in association with Syndecan 4. Interestingly, Schwann cells seeded in functionalized scaffolds with

aligned fibers experienced a significant increase in Syndecan 4 expression (Fig. 9B) from days 3 to 7, in contrast to cells seeded in other scaffolds that did not exhibit significant changes during the same period. Moreover, the aligned functionalized scaffolds achieved an overall higher Syndecan 4 levels on day 7 than cells seeded in aligned nonfunctionalized scaffolds and both random fiber conditions. This shows that a combination of functionalization with GAG and topography of scaffolds is needed for increased expression level of Syndecan 4.

Furthermore, we observed similar patterns comparing protein levels between $\alpha 9\beta 1$ Integrin and Syndecan 1 (Fig. 9C) on days 3 and 7, probably because of their colocalization in the plasma membrane. Laminin showed significantly decreased levels from days 1 to 7 in cells seeded in nonfunctionalized scaffolds with aligned fibers (Fig. 9D). Although the trend was a reduction in the assessed markers during 7 days of culture, an overall increase in protein expression was observed in Schwann cells seeded in functionalized scaffolds (Fig. 3D), both random and aligned, on day 7 of culture, suggesting that, following a lag phase, Schwann cells started to produce ECM proteins themselves. Furthermore, the expression of p75 (or low-affinity nerve growth factor receptor), a Schwann cell marker, was evaluated (Fig. 9E), which showed a considerable decrease from days 1 to 3 and day 7, particularly evident for cells seeded in nonfunctionalized aligned fibers. Overall, all the proteins studied showed a similar trend with increased levels on day 7 in cells seeded in functionalized scaffolds with aligned fibers compared to those of cells seeded on nonfunctionalized scaffolds with aligned fibers (Fig. 3D), even though these data were significant only regarding Syndecan 4.

Fig. 4. Representative immunofluorescence images of Schwann cells seeded on both random and aligned fibers with or without cross-linked GAGs at each time point of cell culture. F-actin is shown in red (Phalloidin-Alexa Fluor 568 staining) and nuclei in blue (DAPI staining). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 5. Representative immunofluorescence images of Schwann cells seeded on both random and aligned fibers with or without cross-linked GAGs at each time point of cell culture. Laminin is shown in purple (Alexa Fluor-647 staining) and nuclei in blue (DAPI staining). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Proteomic profiles

The adopted proteomic approach allowed for the identification of 559 and 872 unique proteins on days 3 and 7, respectively, as shown in [Supplementary Information Tables S1 and S2](#). Comparative analysis evidenced a panel of proteins differentially expressed with a p value < 0.05 and a fold change > 2 ([Supplementary information](#)). Proteomics data compared the 4 conditions on the same day of culture. Results of protein–protein interaction network analysis performed with STRING v10 software showed a greater modulation on GAG-functionalized scaffolds than that of the nonfunctionalized ones, although only few modulated proteins were found comparing fiber orientations. This behavior was summarized in PCA on days 3 ([Fig. 10A](#)) and 7 ([Fig. 10B](#)), where the presence of GAG more than the different alignments of fibers have driven group formation: GAG-functionalized scaffolds grouped closer vs. non-functionalized ones.

On day 3, many binding proteins (TNFR16, NLTP, MAP1B, SSBP, DREB, and SEPT9) were downregulated in GAG-functionalized scaffolds, while other DNA/RNA-binding proteins (RAB14, RS15, HNRH2, and PA2G4), as well as growth regulation protein (PA2G4), were upregulated ([Supplementary Information, Tables S3–S6 and Figs. S5–S8](#)).

The proteomic approach found a greater difference on day 7, with evidence of final effectors indicating a general growing process. No significant difference was detected in cell total protein secretion and proliferation when comparing functionalized and nonfunctionalized scaffolds. Cells grown on GAG-functionalized scaffolds experienced a general downregulation, especially for pro-

teins involved in cell cycle, synthesis, and metabolism ([Supplementary information, Tables S7–S10 and Figs. S9–S12](#)). RG samples on day 7 exhibited a downregulation of transcription factors (PTMA, HP1B3, and HDGR2), translation (EIF3I), and RNA-binding proteins (RL28, RL3, ICAL, CELF1, LPPRC, RL10, and LPPRC), and protein complex assembly (NCLN and NASP) with regard to the corresponding R samples ([Supplementary Information, Table S9, Fig. S11](#)); this is in agreement with the corresponding metabolic and proliferation activity tests. Downregulation of metabolic process and kinase proteins (OLA1, DCLK1, PDXK, NDKB, KAD3, CP51A, AMPL, and NUCKS), as well as cell adhesion molecules (SDC4, 1433B, MARCS, PARVA, and PLPC), was also evident in AG fiber meshes compared to A scaffolds ([Supplementary Information Table S7 Fig. S9](#)). The apparent contrast between the reduced Syndecan 4 (SDC4) detection and the decisive increase shown by western blot can be resolved when considering that Syndecan 4 is a transmembrane protein with an extracellular domain that can be cleaved or ‘shed’ under certain conditions [65]. The western blot data measure the intracellular domain, while the proteomic analysis detects a fragment in the extracellular domain of Syndecan 4; if this domain was already shed at the time of sample preparation, it would not be detected by proteomic analysis. Through this combined analytical approach, we show that Syndecan 4 expression increases and indirectly may reveal an increase in Syndecan 4, shedding in the aligned functionalized scaffold condition. Interestingly, GAG functionalization also resulted in an upregulation of prosaposin, a glycoprotein with reported neurotrophic activity [66], while aligned fibers resulted in an upregulation of lamin-B1 among others ([Supplementary Information Table S8, Fig. S10](#)).

Fig. 6. Representative immunofluorescence images of Schwann cells seeded on both random and aligned fibers with or without cross-linked GAGs at each time point of cell culture. Integrin is shown in green (Alexa Fluor-488 staining) and nuclei in blue (DAPI staining). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

PNI is one of the major clinical problems in restorative medicine and microsurgery, caused primarily by lacerations, fractures, penetrating stretching, or crushing trauma. An estimated 2.8% of trauma patients experience some form of PNI; this number reaches 5% if plexus and root lesions are included [67]. Despite progress in surgical techniques, functional recovery after a serious lesion of a nerve trunk is often incomplete and inadequate.

In contrast with the axons in the central nervous system, peripheral axons have the ability to regenerate. Schwann cells are critical supporting cells of peripheral neurons, participating in neural development, by driving axons to extend to their appropriate terminal organs and promoting nerve repair by facilitating axonal elongation over long distances during axonal regeneration after peripheral nerve injury. In particular, Schwann cells migrate from both proximal and distal nerve stumps to the injury site, where they form the bands of Büngner after Wallerian degeneration [68,69]. Schwann cells also stimulate the elongation of regenerating axons over longer distances by secreting neurotrophins, such as nerve growth factor [70], which are transported to the neural cell body [71].

Several animal studies have shown that early reinnervation of the end organ is of primary importance for an acceptable functional recovery. Delay in repairing an injured nerve represents a huge clinical problem, as it could cause changes that make nerve fibers unable to regenerate, and the muscles undergo atrophy and lose the ability to be reinnervated [72]. Accordingly, researchers are constantly trying to develop strategies to increase the therapeutic success rate of regeneration and functional recovery. In

this aspect, a multidisciplinary approach to solve some of the most demanding medical problems is represented by scaffold-based regenerative medicine strategies, which are trying to match the most suitable synthetic polymer formulations with specific biochemical signals to obtain useful constructs in tissue regeneration.

In this study, electrospun PCL scaffolds were produced using a mandrel. Two different speeds were applied to obtain two types of scaffolds with random or aligned fibers. In both cases, microfibers had a diameter of approximately 1.3 μm , a fiber diameter similar to that of collagen fiber bundles [62]. To investigate the role of GAGs on Schwann cell growth on a polymeric support, scaffolds were functionalized through a cross-linking reaction, which allows GAGs to be covalently bound to the fibers. GAGs are among the major constituents of the ECM and are involved in several physiological and pathological processes [31]. In the PNS, GAGs are known to mediate proliferation, synaptogenesis, neural plasticity, and regeneration [32–34,73]. To evaluate the role of GAGs in nerve regeneration, Schwann cells were seeded on functionalized and nonfunctionalized scaffolds with either random or aligned fibers. After 1, 3, and 7 days of culture, metabolic activity, proliferation, and GAGs assays were performed. Furthermore, the expression of five specific markers was assessed by both immunofluorescence and western blot analyses. Among them, syndecan 1, a transmembrane proteoglycan, is known to be involved in cytoskeletal reorganization and in attachment of cells to ECM, whereas syndecan 4 is known to bind different proteins in ECM and to promote focal adhesion in co-localization with integrins. Additionally, we evaluated the expression of p75, a marker of differentiation in Schwann cells, and of laminin, one of the most abundant ECM proteins in the PNS.

Fig. 7. Representative immunofluorescence images of Schwann cells seeded on both random and aligned fibers with or without cross-linked GAGs at each time point of cell culture. Syndecan 1 is shown in green (Alexa Fluor-488 staining) and nuclei in blue (DAPI staining). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Further, an LC-MSMS-based proteomic profiling approach was chosen to obtain a complete map of Schwann cells grown on non-functionalized and GAG-functionalized scaffolds on days 3 and 7, with day 7 being the only time point with a sufficient number of cells and total protein content for a more robust proteomics profiling. The resulting proteomics library provided a comparison between Schwann cells differentially activated by GAG-functionalized and nonfunctionalized scaffolds.

Overall, cell studies showed that cells increased their metabolic and proliferation activities over 7 days in culture, as well as GAG production. Whereas the alignment of fibers only had a considerable influence on cell morphology, resulting in their elongation, no other marker was affected. Conversely, the functionalization with GAGs resulted in an increase in cell proliferation and GAG formation at very early time points (day 1). There was also a significantly higher expression of Syndecan 4 in GAG-functionalized aligned fibers than in GAG-functionalized random fibers and to nonfunctionalized scaffolds, indicating that both fiber topography and GAG functionalization lead to an increase in Syndecan 4 production.

In general, proteomic analysis revealed a high modulation between conditions for day 7 cultures. Although not statistically significant, a general downregulation of synthetic processes for cells cultured on GAG-functionalized scaffolds was evidenced (RG vs. R, Fig. 3), confirming trends observed in biochemical metabolic and proliferation data. Indeed, a combination of the highly sensitive mass spectrometry technique with more classical biochemical

and immunohistochemical analyses could enable the detection of aspects that could not be otherwise identified.

For example, Syndecan 4 was found to be significantly down-regulated by more than 5-fold in AG than in A, while its expression assayed through western blot analysis seemed to contradict this finding. Syndecan 4 is a transmembrane proteoglycan and is known to bind to a number of ECM molecules and form receptor complexes that bind growth factors and cytokines, subsequently triggering intracellular signaling processes [74]. Syndecan 4 has been shown to influence cell migration and focal adhesion turnover, having a significant role in wound healing and angiogenesis, and it mediates signaling for FGF and other heparin-binding growth factors [74–76]. Furthermore, the expression of Syndecan-4 is increased in response to inflammatory stimuli and this versatile proteoglycan subsequently undergoes “shedding,” whereby the extracellular domain is cleaved and locally modifies the extracellular milieu [77]. Despite having no direct proof in our results that Syndecan-4 shedding has taken place, from what is known in literature, these shed fragments have been shown to block the inhibition of proteases, promoting matrix remodeling during tissue repair, and have been suggested to sequester growth factors, thereby reducing local chemokine concentrations [78,79]. The removal of the Syndecan 4 ectodomain from the cell surface has been shown to promote cell mobility [65]. This implies that this scaffold has the potential to trigger increased cell migration and matrix remodeling, both essential activities for successful nerve repair.

Fig. 8. Representative immunofluorescence images of Schwann cells seeded on both random and aligned fibers with or without cross-linked GAGs at each time point of cell culture. Syndecan 4 is shown in purple (Alexa Fluor-647 staining) and nuclei in blue (DAPI staining). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 9. Histograms showing levels of selected markers expressed by Schwann cells seeded in the four constructs during 7 days of culture, obtained by western blot analysis. (A) Integrin. (B) Syndecan 4. (C) Syndecan 1. (D) Laminin. (E) p75. Data are normalized for β -actin expression. *p < 0.05, **p < 0.01.

Fig. 10. Principal Component Analysis (PCA) at days 3 (panel A) and 7 (panel B). PCA is a statistical analysis that gives a score to each sample (axes represent the scores) so to represent how much the samples are far or close to each other. Close samples means that their profiles are similar.

Prosaposin (SAP) and Tubulin-specific chaperone A (TBCA) upregulation in AG and RG, respectively, reflected glycoprotein increase in functionalized samples already seen on day 7 for AG samples in GAG assay (Fig. 3). SAP, in particular, is a known neurotrophic factor that has been shown to promote peripheral nerve repair [80]. Myristoylated alanine-rich C-kinase substrate (MARCKS) plays important roles in cell shape, cell motility,

secretion, transmembrane transport, regulation of the cell cycle, and neural development [81].

Cell adhesion molecules were downregulated in functionalized samples, mirroring the general trend of reduction of the assessed markers during 7 days of culture (Fig. 9). The reduced expression of HDG2, known to promote cell growth [82] and DNA repair [83], further corroborates data from proliferation assay for RG

samples. Similarly, the observed reduction in metabolic activity was reflected in the downregulation of CP51A, a cytochrome P450 isoenzyme that serves in the metabolism and biosynthesis of steroids, lipids, and vitamins.

In this study, we have focused on early response of Schwann cells to GAGs motifs functionalized on the surface of microfibrillar meshes. Future studies will have to investigate longer culturing times to better assess the potential of GAG-functionalized scaffolds for neural regeneration. Such studies will have to be followed by validation *in vivo*, where it will be also important to assess the influence of the local inflammatory microenvironment. It is known, in fact, that macrophages play a pivotal role in eliminating inhibitory substances from the nerve regenerative microenvironment [84]. Altogether, our results suggest that GAG-functionalized scaffolds may have the potential to create an extracellular environment similar to that of the natural Schwann cell basal lamina. Moreover, increased levels of Syndecan 4 in functionalized scaffolds with aligned fibers indicate that this proteoglycan may lead Schwann cells to a localized ECM environment after nerve injury where ECM proteins, such as fibrin, form cables capable to conduct nerve regeneration [85].

5. Conclusions

Although nerve fibers of the PNS have a remarkable ability to regenerate because of primarily Schwann cells, post-traumatic functional recovery after a severe lesion of a major nerve trunk is often incomplete and unsatisfactory. In this aspect, a major challenge of tissue engineering is to develop a synthetic scaffold with both mechanical and biochemical properties (i.e., biocompatibility, controlled biodegradability with nontoxic products, low inflammatory potential, and porosity) that promotes cell attachment and growth during the regenerative process.

Actually, several synthetic polymers and natural materials are used to fabricate scaffolds with the required properties by electrospinning, nanofiber self-assembly, liquid–liquid phase separation, solvent casting and particulate leaching, emulsification/freezing-drying, gas foaming, and additive manufacturing. Biocompatibility of engineered scaffolds is often enhanced by surface modification of the material, in particular, by attaching bioactive molecules.

Two well-defined fiber deposition patterns were presented (aligned or random fibers), which we successfully functionalized with GAGs purified from aorta porcine tissue and assessed for their potential as support for Schwann cell adhesion, growth, and differentiation, by both classical biochemistry and LC-MS-based proteomic profiling. The latter allowed to obtain a complete protein profile of Schwann cells as well as to identify differentially expressed proteins between cells grown on different constructs. Comparing results obtained from these quite different approaches allowed us to show that PCL–GAG scaffolds could represent a promising artificial substrate that closely mimics the recently established pattern of Schwann cell migration into the regenerating nerve.

Data availability

Data are available from the corresponding author, upon request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.actbio.2019.06.054>.

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