Nerve guidance conduits based on bi-layer chitosan membranes for peripheral nerve regeneration

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Abstract

Chitosan (CS), a derivative of the naturally occurring biopolymer chitin, has been widely used in a variety of biomedical applications including peripheral nerve repair because of its excellent biocompatibility, biodegradability, readily availability and antibacterial activity. In this work, bi-layer CS flat membranes were developed to be easily manipulated and rolled to obtain flexible nerve guidance channels (NGCs). These bi-layer membranes were prepared via solvent casting technique and their chemical composition was previously optimized to realize flexible membranes to be enwrapped for NGC formation. Two kinds of CS films cross-linked with different crosslinking agents were combined to produce scaffold structures with both good biocompatibility and the desired mechanical strength imparted by the inner and the outer layer, respectively. A tight connection between the two layers was achieved and the physicochemical and mechanical properties of CS bi-layer membranes were investigated. Additionally, bi-layer membranes were used for bridge implantation across 10-mm long median nerve defects in rats, and the outcome of peripheral nerve repair at 12 weeks post-implantation was evaluated by a combination of immunohistochemical and histological investigations.

Introduction

Despite over 150 years of experience in modern surgical management of the peripheral nerve, repair of a nerve gap remains a significant problem in microsurgery.[1] Usually, peripheral nerve injuries that result in gaps longer than 40 mm require surgical implantation of a bridge or guidance channel between the proximal nerve end and the distal stump in order to restore full function and organ regeneration.[2] Autologous nerve grafts is the *gold standard* technique commonly used in bridging peripheral nerve defects.[3] However, there are unavoidable drawbacks associated to it such as their limited availability, the donor-site morbidity, the mismatch in size and the necessity for multiple surgeries.[4] Although allografts have also been used, these must be accompanied by immunosuppression therapy and have a lower success rate than autograft.[5] The development of alternative treatments is therefore highly desirable. Artificial nerve guidance channels (NGCs) can create a favorable microenvironment by mimicking the structure and composition of an autograft and might potentially match the autograft performance in terms of regeneration capacity.[6]

Until now, a variety of biomaterials have been developed for the repair of peripheral nerve injuries, including several natural and synthetic polymers.[7,8] Chitosan (CS), as a natural polysaccharide, has attracted increasing attention due to its good biocompatibility, biodegradability, non-toxicity, readily availability and unique physicochemical properties.[9-11] Recent *in vitro* studies revealed the suitability of CS membranes as a substrate for survival and oriented growth of Schwann cells[12] as well as survival and differentiation of neuronal cells.[13,14] Yang and colleagues immobilized nerve growth factor (NGF) on CS scaffolds and found that the NGF release could be controlled by CS scaffolds.[15] In addition, CS NGCs alone or in combination with other biomaterials can efficiently bridge peripheral nerve gaps.[16-18] Despite the interesting biological properties of CS scaffolds, its applicability in several surgical approaches is limited by the poor mechanical strength under physiological conditions and the incapacity to maintain a predefined shape for transplantation.[19,20] Improved technologies and different crosslinking methods have been developed to improve the mechanical properties of CS NGCs under physiological conditions.[21,22] CS membranes silanized with γ-glycidoxypropyltrimethoxysilane (GPTMS) have been found to significantly improve post-traumatic axonal re-growth and functional recovery.[23] Recently, Ruini and colleagues compared the effects of different covalent and ionic crosslinking agents on CS physico-chemical and mechanical properties.[22] According to the obtained results, CS membranes, crosslinked with dibasic sodium phosphate (DSP) alone or in association with GPTMS seem to have suitable physico-chemical and mechanical properties for peripheral nerve tissue engineering.

In this study, a bi-layer CS flat membrane with CS/DSP film as inner layer and CS/GPTMS_DSP as outer layer was developed. The two layers were selected on the basis of results previously reported by authors.[22] The composition of the bi-layer membrane was selected since CS/DSP demonstrated to provide a substrate to promote cell adhesion, proliferation and migration as a template to guide the formation of new neural tissue and the CS/GPTMS_DSP membrane could act as a temporary scaffold, imparting the desired mechanical strength to the conduits. The obtained bi-layer membranes were analyzed for their physicochemical properties by Fourier transform-infrared spectroscopy (FTIR) analysis and static contact angle measurements, their water stability was studied by swelling and dissolution tests in phosphate buffer solution (PBS), their mechanical properties were evaluated by tensile tests. Finally, conduits were fabricated by rolling the developed membranes

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Contributions: FR and CT-T were involved in the conception and design of the work; FR analysed and interpreted the data for what concerns physico-chemical characterization, mechanical analysis, as well as permeability, swelling and dissolution tests. CT-T contributed to the drafting of the article; GC critically revised the article regarding intellectual content; SR performed the *in vivo* characterization and contributed to the analysis and interpretation of the data for *in vivo* characterization; SG critically revised the article concerning *in vivo* tests; PT and PP performed the surgical interventions on animals; GC and SG approved the version to be submitted.

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and used for preliminary *in vivo* studies. Bi-layered conduits were applied to bridge the 10 mm defects in the median nerve of female Wistar rats, and autograft nerves were taken as positive controls.

Materials and Methods

Chitosan (medium molecular weight, 75-85% deacetylation degree), GPTMS and DSP were supplied from Sigma Aldrich (St. Louis, MO, USA). All solvents used were of analytical grade.

Chitosan bi-layered scaffolds were produced by adopting a two-step coating technique. CS was dissolved in a 0.5 M acetic acid solution at room temperature by continuous stirring to obtain a 2.5% (wt./vol.) solution. First, the inner layer was prepared by adding 1M DSP to the CS solution (CS/DSP) with a concentration of 7.5% vol./vol. with respect to the natural polymer solution volume, as previously reported.[22] The mixed solution was kept under stirring at room temperature for about 10 minutes. Then, 10 mL of CS/DSP solution was poured into 6 cm Petri dishes and air-dried for 48 hours to obtain inner layer membrane. Later, external layer was prepared by adding GPTMS (25% wt./wt.) to the CS solution. The resulting CS/GPTMS solution was kept under stirring for 1 hour, followed by the dropwise addition (one drop per second) of DSP 1M (concentration 7.5% vol./vol.) and maintained under moderate stirring for 10 minutes. Finally, 10 mL of CS/GPTMS_DSP solution was poured directly on top of the previously dried layer and air-dried for 48 hours. For the realization of the CS/GPTMS_DSP external layer, the amount of GPTMS was decreased from 50 to 25% wt./wt. with respect to flat membranes prepared in a previous work with the final aim to reduce compositional differences and optimize the adhesion among the inner and outer side in the bi-layer structure.[22] Single layer CS flat membranes were prepared as control following the procedures described above by pouring 10 mL of CS/DSP and CS/GPTMS_DSP solutions on Petri dishes (diameter 6 cm).

Sample characterization

Fourier transform infrared-attenuated total reflectance spectroscopy

The FTIR-attenuated total reflectance spectroscopy (FTIR-ATR) spectra of CS/GPTMS_DSP, CS/DSP and bi-layer samples were recorded at room temperature in a Perkin Elmer Spectrometer in the range 2000-600 cm^{-1} at a resolution of 4 cm^{-1}.

Surface wettability

The static contact angle of CS/DSP, CS/GPTMS_DSP and bi-layer films were measured at room temperature using a KSV instrument equipped with a CAM 200 software for data acquisition. The sessile drop method was applied, using a 5 μL double distilled water droplet. For each angle reported, ten measurements on different surface locations were measured and results were expressed as average value ± standard deviation.

Mechanical properties

The tensile mechanical properties were performed on wet flat membranes using MTS QTest/10 device equipped with load cells of 10 N. Rectangular strips (30x10 mm) were cut from each sample and strained to break at a constant crosshead speed of 2 mm/min. Prior to tensile testing, films were immersed in PBS for 10 minutes at 25°C. Using the associated software Test Works, break stress and strain were determined. The elastic modulus was calculated from the slope of the linear portion of the stress–strain curve. A digital calibrator was used to measure the thickness of the films. Four specimens for each kind of material were tested. The results were expressed as average values±standard deviation.

Swelling and dissolution tests

The swelling and dissolution behavior of single and double layer samples were evaluated by immerging the samples in PBS (pH 7.4) at 37°C. The swelling degree was measured after 1, 3, 6, 9 and 24 hours while the dissolution degree was evaluated at time intervals of 1, 3, 5, 7, 14, 28 and 56 days. The swelling percentage was calculated as:

$$\Delta W_s\,(\%) = (W_s - W_0)/W_0 * 100$$

where W_0 and W_S are the sample weights before and after swelling, respectively. The total dissolution percentage was calculated as:

$$\Delta W_d\,(\%) = (W_0 - W_d)/W_0 * 100$$

where W_d is the dried sample weight after dissolution. The solution pH was measured at the same time intervals during the swelling and the dissolution tests, and its stable value at around physiological pH was verified. For each experimental time, three samples were measured and results were expressed as averages value±standard deviation.

Permeability of bi-layer chitosan based membranes

Permeability of CS/DSP, CS/GPTMS_DSP and bi-layer membranes to fluorescein isothiocyanate (FITC)-labeled dextrans (Sigma Aldrich) of 4400 Da (FD-4) was determined according to the procedure followed by Ruini and colleagues.[22] Briefly, samples were rolled and glued with a cyanoacrylate glue to obtain a tube closed at one end. A 10% wt./vol. solution

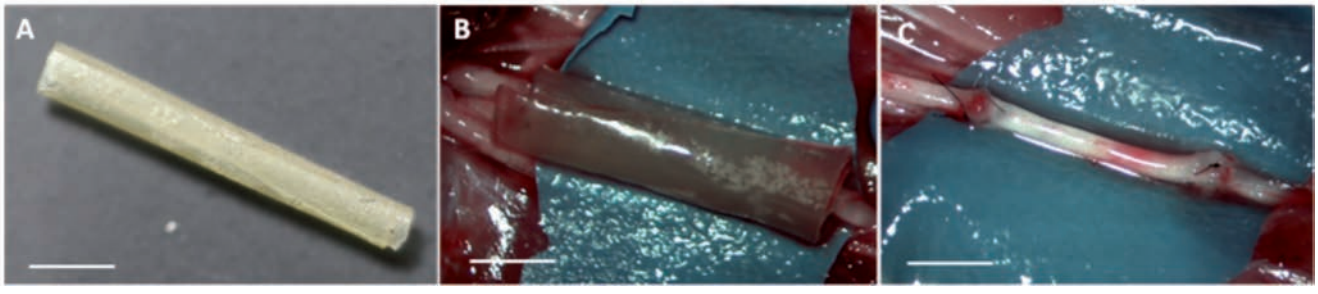

Figure 1. Preliminary *in vivo* tests. Bi-layer membrane enrolled and glued with a cyanoacrylate glue to prepare a nerve guidance channel for repairing severe median nerve lesions (A). Nerve guidance channel (B) and autograft (C) immediately after implantation for the repair of a rat median nerve. Scale bar: 3 mm.

of FD-4 in PBS was prepared, and 180 µL of the solution were inserted into the lumen, then the second opening was closed with the cyanoacrylate glue. The tube was then placed in 3 mL of PBS at pH 7.4, and FITC-dextran concentration in the incubation medium was assayed fluorimetrically (CARY 500 SCAN UV-VIS-NIR Spectrophotometer) after 1, 3, 6, 24, 48 and 96 hours. The FD-4 concentration was calculated from the absorption values using the calibration curves obtained from FD-4 solution of known concentrations and was reported as a percentage of the initial concentration (10% wt./vol.) in the tube. Five measurements for each sample were taken and data reported as mean value with its standard deviation.

Preliminary *in vivo* tests

All procedures were performed in accordance with the Ethics Committee and the European Communities Council Directive of 24 November 1986 (86/609/ EEC).

Animals and surgery

In vivo nerve regeneration assay was carried out with bi-layer membranes (Figure 1A,B) and the autograft was used as control (Figure 1C). A total of 8 adult female Wistar rats weighing approximately 250 g at the start of the experiment were used. The animals were divided in two experimental groups of 4 animals each. In the experimental group the median nerve was transected and encircled with bi-layer conduits (Figure 1B) while in the control group the median nerve was repaired with reversed autologous nerve graft (Figure 1C). Before surgery, bi-layer membranes were immersed in PBS solution, rolled up and glued with biomedical cyanoacrylate glue to obtain a tube having a 1.1 mm diameter and a 12 mm length (Figure 1A). The surgery procedure was the one previously described by Tos and colleagues.[24] After 12-weeks post-operative, rats were sacrificed and regenerated nerves were analysed by light and electron microscopy.

Resin embedding and transmission electron microscopy

After the 12-week follow-up time, animals were euthanatized and a 10-mm long segment of the median nerve distal to the site of lesion was collected, fixed, and prepared for electron microscopy. Nerve samples were fixed by immediate immersion in 2.5% purified glutaraldehyde and 0.5% saccarose in 0.1 M Sorensen phosphate buffer for 6-8 hours. Specimens were then washed in a solution containing 1.5% saccarose in 0.1 M Sorensen phosphate buffer, post-fixed in 1% osmium tetroxide, dehydrated and embedded in resin.

From each nerve, series of ultra-thin transverse sections (50-90-nm thickness) were cut starting from the distal stump of each median nerve specimen, using an Ultracut UCT ultramicrotome (Leica Microsystems, Wetzlar, Germany) and stained using saturated aqueous solution of uranyl acetate and lead citrate for transmission electron microscopy (JEOL, Tokyo, Japan).

Immunohistochemistry and confocal laser microscopy

The conduit of all animals was frozen immediately after the harvesting. Longitudinal sections of 10 m were cut by cryostat (Leica Microsystems) and processed for immunohistochemistry and confocal laser microscopy. Sections were then incubated overnight in a solution containing -NF-200 kD primary antibody (monoclonal, mouse, which recognizes the 200 kD subunit of neurofilaments, dilution 1:200; Sigma) and then, after washing in PBS, incubated for 1 hour in a solution containing ALEXA 488-conjugated anti-mouse IgG (dilution 1:200; Dako). The sections were finally mounted with a Dako fluorescent mounting medium and analyzed by a LSM 510 confocal laser microscopy system (Zeiss, Jena, Germany), which incorporates two lasers (Argon and HeNe) and is equipped with an inverted Axiovert 100 M microscope.

Statistics

Statistical analysis was carried out using single-factor analysis of variance (ANOVA) post hoc Bonferroni. Values of *P≤0.05, **P≤ 0.01, ***P≤0.001 were considered as statistically significant.

Results and Discussion

Fourier transform infrared-attenuated total reflectance spectroscopy

The FTIR-ATR spectra of CS based film sam-

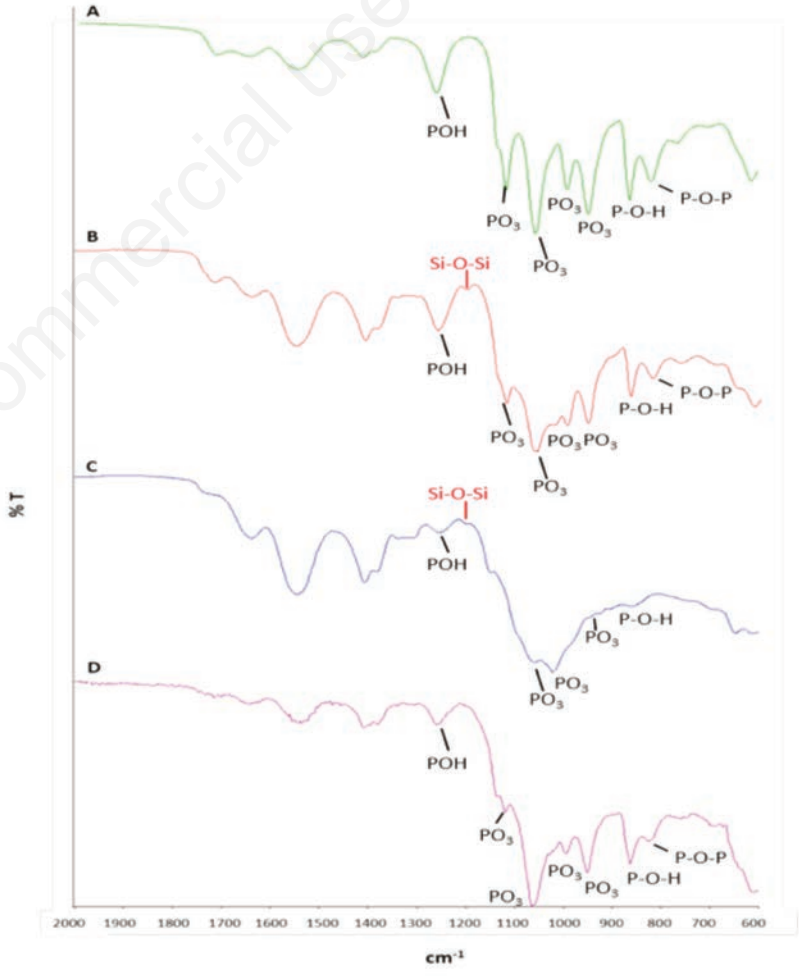

Figure 2. Fourier transform-infrared spectroscopy-attenuated total reflectance spectroscopy spectra of chitosan/dibasic sodium phosphate (A), chitosan/γ-glycidoxypropyltrimethoxysilane_dibasic sodium phosphate (B), external (C) and internal side (D) bilayer membrane.

ples are reported in Figure 2. CS/DSP, CS/GPTMS_DSP, internal and external side bi-layer spectra showed the characteristics bands of both CS and DSP. In details, the peak at 1674 cm^{-1}, 1544 $cm^{-1}$and 1414 cm^{-1} were associated to the C=O stretching bond, the amide and amine bending vibrations and O-H bending vibrations typical of CS, respectively. DSP crosslinking was confirmed by the detection of bands at 1000 cm^{-1} and at 989 cm^{-1} and 943 cm^{-1} due to the PO_3 out-of-phase and in-phase stretching; typical absorption peaks at 861 cm^{-1} and at 814 cm^{-1} were associated with P-OH stretching and P-O-P asymmetric stretching vibration, respectively.[25] Moreover, the CS/GPTMS_DSP and external side of the bi-layer FTIR-ATR spectrum showed bands at 1196 cm^{-1} due to the Si-O-Si bonds of the covalent crosslinking chains, confirming the successful crosslinking of CS by GPTMS. The absorption band at 1720 cm^{-1} was observed in all spectra and could be due to acetic residuals (stretching vibration of C=O groups).[26]

Surface wettability

The static water contact angles of model CS/DSP, CS/GPTMS_DSP, internal and external side of bi-layer samples are reported in Figure 3. The average contact angles of CS/DSP and CS/GPTMS_DSP were 66°±9° and 65°±11°, respectively. The development of a double layer flat membrane significantly modified the wettability of the inner layer as compared to CS/DSP and CS/GPTMS_DSP films (**P<0.01). Static water contact angles of the internal and external side of the bi-layer samples were also significantly different (41°±9° for inner and 64°±16° for outer layer; **P<0.01).

The enhanced hydrophilicity of the internal part of the double layer membrane has to be ascribed to the physicochemical interactions between the inner and outer layer that occur during the fabrication process that increased the amount of exposed phosphate groups and consequently the surface wettability. The augmented hydrophilic nature of the bi-layer flat membrane is favorable for improved cell attachment on the materials developed.[27,28]

Mechanical properties

CS based samples were tested in the wet state in order to reproduce the conditions during practical use. All specimens showed an elasto-plastic behavior; the ϛ-ε slope was calculated to obtain the elastic modulus. Wet CS/GPTMS_DSP and CS/DSP samples had a uniform thickness in the range of 90-130 m, while bi-layer membrane showed thickness values within 300-350 m. The presence of GPTMS enhanced the E values (E, from 3.47±1.06 MPa for CS/DSP to 9.29±0.85 MPa and 11.01±2.08 MPa for CS/GPTMS_DSP and bi-layer, respectively): the increase of the elastic modulus of CS/GPTMS_DSP and bi-layer samples (statistical significant compared to CS/DSP samples, **P<0.01 and ***P<0.001) was a consequence of the mechanical reinforcement associated with covalent crosslinking. Additionally, results obtained indicated the increased elasticity of CS/GPTMS_DSP and of double layer membranes compared to the ionic cross-linked samples. No significant differences were observed between CS/GPTMS_DSP and bi-layer samples. Moreover, the developed bi-layer samples showed an intermediate behavior in terms of elongation at break compared to CS/GPTMS_DSP and CS/DSP (Figure 4), indicating that the double layer have intermediate properties resulting from the combination of the CS/DSP internal side and CS/GPTMS_DSP external side.

Biomaterials used to fabricate NGCs are expected to possess mechanical flexibility to favor their surgical application and the permanence in the body without collapsing under compression during axonal outgrowth. The elastic modulus of the mouse sciatic nerve has been reported to be 7 MPa.[29] A nerve guide able to mimic the behavior of the natural nerve tissue should possess a similar elastic modulus. In this work, elastic modulus values of around 10 MPa were measured for CS/GPTMS_DSP and bi-layer samples and showed superior mechanical properties respect to CS/DSP samples. Moreover, the development of bi-layer membranes allowed increasing the elongation at break compared with CS/GPTMS_DSP. Additionally, all the cross-linked scaffolds could be easily wrapped around the trunked nerve stumps, allowing the use of the different typologies of membranes for the preparation of NGCs during the surgical intervention and with the appropriate diameter size depending on the treated nerve size (data not shown).

Swelling and dissolution tests

The CS based membranes prepared by solvent casting increased their weight when immersed in PBS and after 1 hour the water

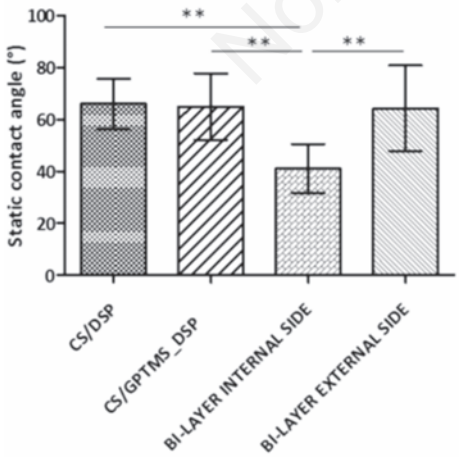

Figure 3. Static water contact angle of chitosan/dibasic sodium phosphate (CS/DSP), chitosan/γ-glycidoxypropyltrimethoxysilane_dibasic sodium phosphate (CS/GPTMS_DSP), internal and external side of bi-layered film samples. Histograms reported the average values and the standard deviations. **P<0.01.

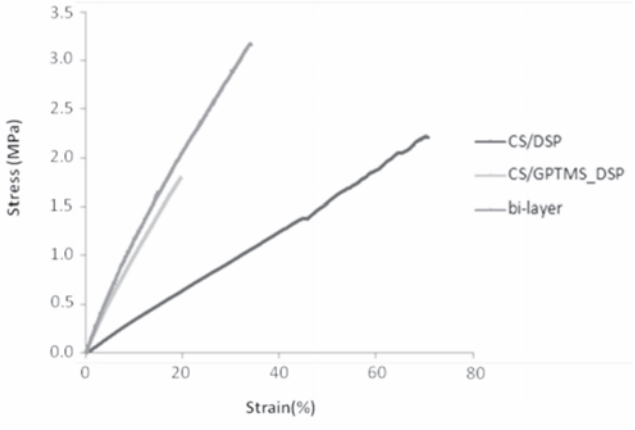

Figure 4. Stress-strain curves of chitosan/dibasic sodium phosphate (CS/DSP), chitosan/γ-glycidoxypropyltrime-thoxysilane_dibasic sodium phosphate (CS/GPTMS_DSP), and bi-layer flat membranes tested in wet state.

uptake was 532±20, 76±19 and 140±5% for CS/DSP, CS/GPTMS_DSP and bi-layer samples, respectively (Figure 5A). The CS/GPTMS_DSP and CS/DSP swelling values stabilized after 3 hours, while a slightly and continuous increase was measured for the bi-layer membranes reaching a water uptake value of around 293±67% after 24 hours. According to the previous results obtained by the authors, covalent and/or ionic crosslinking seems not to affect the swelling kinetic profile of CS based samples.[22] In this work, a gradual increase in swelling degree was observed for the bi-layer membranes and could be ascribed to the higher thickness of the double layer samples compared to single layer which slows down the water absorption in time.

Moreover, an intermediate value of water uptake for the bi-layer membranes was observed as a result of the combination of single component behaviour: the high swelling degree caused by the presence of phosphate groups for the inner layer and the moderate water absorption capacity of the external layer due to the formation of a well-organized structure following GPTMS crosslinking. A comparison of the swelling percentage of CS based samples revealed that the water uptake of CS/DSP samples was significantly higher than for CS/GPTMS_DSP and bi-layer specimens at each time point (***P<0.001).

The dissolution profiles of CS based samples after 56 days of immersion in PBS are presented in Figure 5B. All membranes decreased their weight of 44.6±5.6, 34.5±1.9 and 38.2±5.2% for the CS/DSP, the CS/GPTMS_DSP and the bi-layer after 1-day incubation in PBS. CS/GPTMS_DSP samples showed significant lower weight loss with respect to CS/DSP specimens at this time point. The initial high weight loss was associated to the release of salts into PBS solution, as confirmed by EDS spectra and elemental mapping (S1). CS/DSP, CS/GPTMS_DSP and the weight loss of the bi-layer reached final values of 67.3±6.6, 54.7±1.5 and 54.2±3.6% after 56 days incubation in PBS. Compared to the CS/DSP membranes, chemical crosslinking by the addition of GPTMS agent enhanced the stability in aqueous media of CS/GPTMS_DSP and bi-layer membranes.

Biomaterials applied for NGCs should degrade within the nerve regeneration period, which depends on the extent of lesion (2-5 mm/day axonal growth rate).[7] During this period, NGC should provide an isolated microenvironment avoiding the risk of fibroblast infiltration and providing a conduit wall for the exchange of fluid and nutrients. The high weight dissolution degree of the developed membranes after 1h of immersion in PBS did not affect the structure of the membranes thus ensuring adequate conditions for peripheral nerve regeneration.

Permeability of bi-layer chitosan based membranes

Figure 6 reports the release concentration of FD-4 in PBS after 1, 3, 6, 24, 48 and 96 hours. The FD-4 was used as a model of nutrients having a Stokes radius of 14 Å that is superior to glucose (3.8 Å) and NaCl Stokes radius (1.4 Å).[30] Results showed that the different structures (single or bi-layer) influenced FD-4 release kinetics. The release of FD-4 from CS/DSP and CS/GPTMS_DSP tubes had an initial burst release (about 50 and 65% for CS/DSP and CS/GPTMS_DSP, respectively) from the inside to the outside of conduits after 1 hour of the samples immersion in PBS. Then, a sustained release stage of FD-4 contained into CS tubes was observed reaching a final value of around 80% of model molecule released in 96 hours for both the single layer samples. No significant differences were observed between CS/DSP and CS/GPTMS_DSP membranes at each time point. Concerning the bi-layer samples, a more controlled release of FD-4 was observed with time. In detail, multi-layer tubes released about 28% of the model molecule in the first 6 hours of incubation in PBS. Then, a burst release was observed for 24 hours reaching a FD-4 release value of 72±3% after 4 days (96 hours). This behavior can be ascribed to the higher thickness of the double layer specimens compared to CS/DSP and CS/GPTMS_DSP conduits which slows down the penetration of FD-4 inside the bi-layer tubes after PBS absorption during CS swelling, as also evidenced by swelling tests. However, no significant differ-

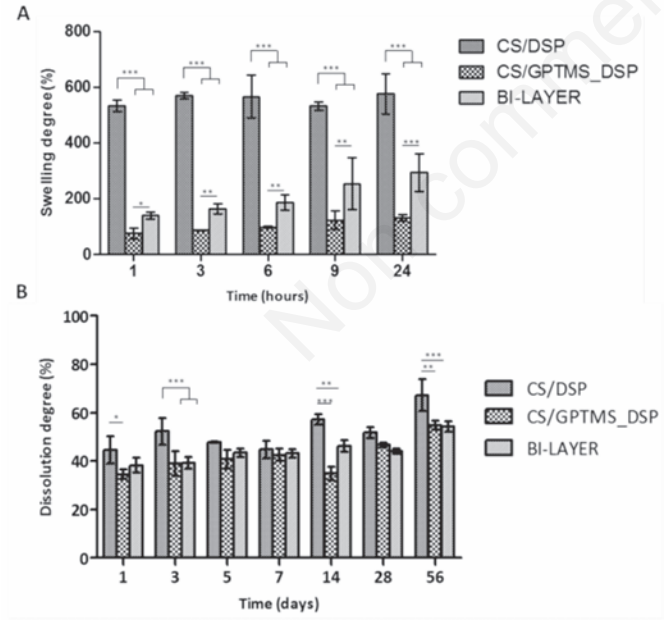

Figure 5. Swelling (A) and total dissolution (B) percentages of chitosan/dibasic sodium phosphate (CS/DSP), chitosan/γ-glycidoxypropyltrimethoxysilane_dibasic sodium phosphate (CS/GPTMS_DSP), and bi-layer flat membranes in phosphate buffer solution as a function of time. Column heights correspond to the mean values. Bars indicate standard deviations (n=3). *P<0.05, **P<0.01, ***P<0.001.

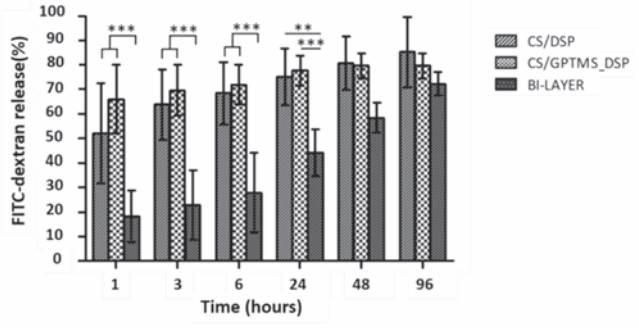

Figure 6. Fluorescein isothiocyanate-dextran concentration in the incubation medium reported as a percentage of the dextran initially loaded into the tube. Columns are the average values; bars represent the standard deviation (n=4). *P<0.05, **P <0.01, ***P<0.001.

ence of total model molecule release was observed between the different typologies of structure (single and bi-layer) after 96 hours of immersion in PBS confirming the permeability of the bi-layer membrane for solutes and nutrients, such as NaCl and glucose.

Preliminary *in vivo* tests

Transmission electron microscope analysis

Figure 7 shows electron microscope images of distal part of rat median nerves injured, repaired with reversed autologous nerve graft (Figure 7 A,C,E) or bi-layer conduits (Figure 7 B,D,F) and harvested at 12 weeks after surgery. Small myelinated axons, at different myelination stages, unmyelinated fibers and microfasciculation typical of regenerated nerve fibers, due to the perineural cells activity, were detected in both experimental groups on all treated animals through transmission electron microscopy observation.

Immunohistochemistry and confocal laser microscopy

Axonal regeneration onto bi-layer conduits was examined by confocal laser microscopy on longitudinal nerve frozen sections after NF staining (Figure 8). After 12-week post-operative, a densely population of NF axon alignment was observed for bi-layer membranes (Figure 8). These results confirm that the presence of CS/DSP internal side of the bi-layer structure could be beneficial for axon regeneration from the proximal to the distal stump.

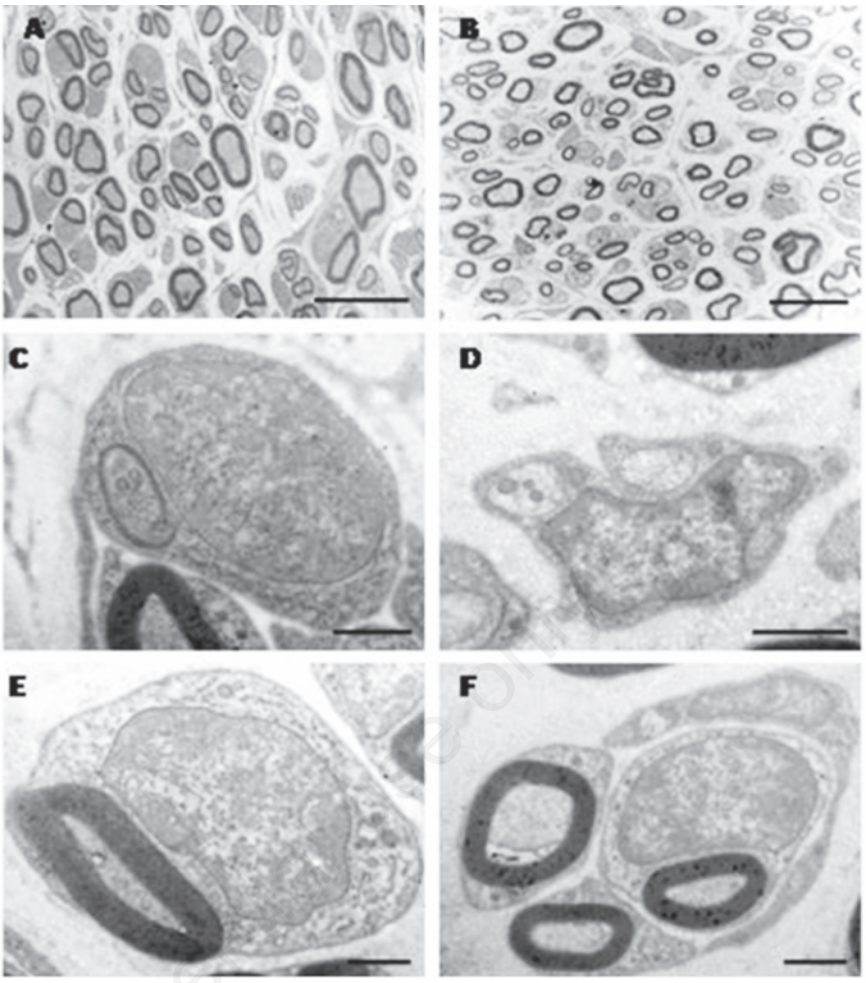

Figure 7. Electron microscope images of regenerated nerves repaired using autologous nerve graft (A, C, E) and bi-layer membranes (B, D, F) after 12 weeks post-operative. Scale bars: 10 μm (A, B), 1 μm (C-F).

Conclusions

NGCs are a promising alternative for autologous nerve graft repair to enhance the regeneration of small nerve gaps. In the present study, bi-layer flat membranes were studied *in vitro* and *in vivo* for the development of CS based nerve conduit scaffolds. The double-layered membranes were fabricated by a two-step coating technique and they could be easily rolled to form a NGC in wet state.

The physico-chemical characterization showed that the two single layers composing the developed membrane interact during the double-layer fabrication process: an increase in the surface wettability of the structure was observed and could be associated to the strong hydrogen bonding and the higher amount of phosphate groups in the bi-layer membrane [(CS/DSP+CS/GPTMS_DSP compared to a single layer membrane (CS/DSP)]. The bi-layer samples tested under wet condition showed improved mechanical properties compared with the CS/DSP and a higher elongation at

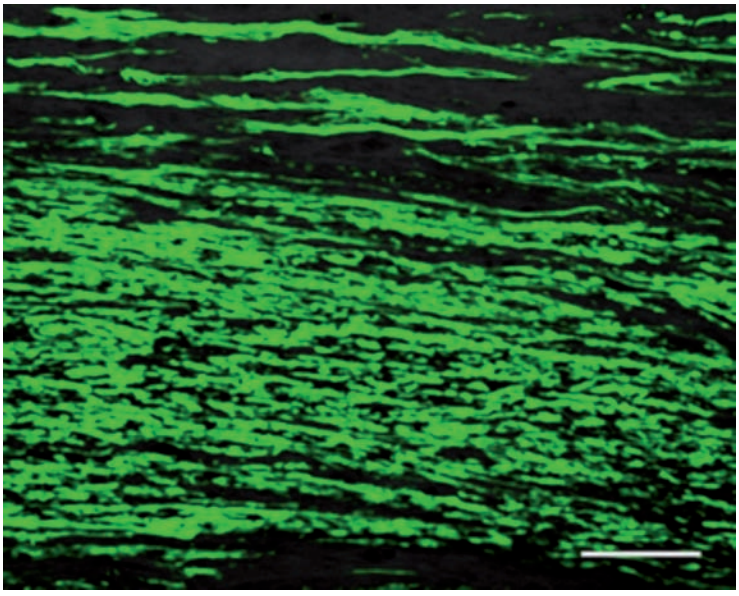

Figure 8. Neurofilament staining on longitudinal sections at 12 weeks revealed that bi-layers were densely populated with axons properly, linearly aligned. Scale bar: 100 μm.

break of CS/DSP film was observed as well. Bi-layer samples showed intermediate swelling degree compared to the single layers and the permeation to small molecules from the inside to the outside of the by-layered membranes was confirmed. Finally, preliminary *in vivo* tests were carried out on the bi-layers conduits for bridge implantation across 10-mm long median nerve defects in rats. After 12 weeks post-operative, nerves repaired with bi-layer tubes displayed regenerated and aligned fibers inside the conduit.

These promising results indicate that double-layer CS based conduits should deserve investigation as a tool for promoting peripheral nerve regeneration.

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