ARTIGO ORIGINAL

Acta Med Port 2011; 24: 043-052

IN VITRO AND IN VIVO CHITOSAN MEMBRANES TESTING
For Peripheral Nerve Reconstruction

M.J. SIMÕES, A. GÄRTNER, Y. SHIROSAKI, R.M. GIL DA COSTA, P.P. CORTEZ, F. GARTNËR, J.D. SANTOS, M.A. LOPES, S. GEUNA, A.S.P. VAREJÃO, A. Colette MAURÍCIO

RESUMO

Tissue regeneration over a large defect with a subsequent satisfactory functional recovery still stands as a major problem in areas such as nerve regeneration or bone healing. The routine technique for the reconstruction of a nerve gap is the use of autologous nerve grafting, but still with severe complications. Over the last decades several attempts have been made to overcome this problem by using biomaterials as scaffolds for guided tissue regeneration. Despite the wide range of biomaterials available, functional recovery after a serious nerve injury is still far from acceptable. Prior to the use of a new biomaterial on healing tissues, an evaluation of the host's inflammatory response is mandatory. In this study, three chitosan membranes were tested *in vitro* and *in vivo* for later use as nerve guides for the reconstruction of peripheral nerves submitted to axonotmesis or neurotmesis lesions. Chitosan membranes, with different compositions, were tested *in vitro*, with a nerve growth factor cellular producing system, N1E-115 cell line, cultured over each of the three membranes and differentiated for 48h in the presence of 1.5% of DMSO. The intracellular calcium concentrations of the non-differentiated and of the 48h-differentiated cells cultured on the three types of the chitosan membranes were measured to determine the cell culture viability. *In vivo*, the chitosan membranes were implanted subcutaneously in a rat model, and histological evaluations were performed from material retrieved on weeks 1, 2, 4 and 8 after implantation. The three types of chitosan membranes were a viable substrate for the N1E-115 cell multiplication, survival and differentiation. Furthermore, the *in vivo* studies suggested that these chitosan membranes are promising candidates as a supporting material for tissue engineering applications on the peripheral nerve, possibly owing to their porous structure, their chemical modifications and high affinity to cellular systems.

MJ.S., A.G., P.P.C., R.M., F.G., A.C.M.: Centro de Estudos de Ciência Animal. Instituto de Ciências e Tecnologias Agrárias e Agro-Alimentares. Universidade do Porto. Porto
M.J.S., A.G., P.P.C., R.M.G.C., F.G., A.C.M.: Instituto de Ciências Biomédicas de Abel Salazar. Universidade do Porto. Porto
Y.S., JDS., M.A.L.: Secção de Materiais. Faculdade de Engenharia. Universidade do Porto. Porto
Y.S.: Division of Functional Molecular Chemistry, Graduate School of Natural Science and Technology. Research Centre for Biomedical Engineering. Okayama University. Tsushima, Okayama. Japan
S.G.: Dipartimento di Scienze Cliniche e Biologiche. Università di Torino. Italy
F.G.: Instituto de Patologia e Imunologia Molecular da Universidade do Porto. Porto
A.S.P.V.: Departamento de Ciências Veterinárias. Universidade de Trás-os-Montes e Alto Douro. Vila Real

SUMMARY

AVALIAÇÃO *IN VIVO* E *IN VITRO* DE MEMBRANAS DE QUITOSANO
Para Utilização em Reconstrução de Nervo Periférico

A regeneração de tecidos em situações clínicas que conduzem à formação de grandes defeitos, com subsequente recuperação funcional continua a ser um grande desafio, principalmente no que diz respeito ao tecido ósseo e tecido nervoso periférico. A técnica cirúrgica de rotina para reconstrução de um nervo periférico após secção e/ou perda de substância é a sutura topo-a-topo de um segmento de nervo autólogo (auto-enxerto), o que conduz a várias possíveis complicações. Durante as últimas décadas, têm sido investigados vários biomateriais (naturais ou sintéticos) com o objectivo de servirem de estrutura à regeneração de tecidos. Apesar da grande variedade de biomateriais disponíveis, a

Recebido em: 13 de Agosto de 2009
Aceite em: 25 de Setembro de 2009

recuperação funcional após uma grave lesão de um nervo periférico continua a ser um grande desafio para a neurologia, com resultados muitas vezes pouco aceitáveis. Antes de se utilizar um biomaterial em clínica, é obrigatório o seu estudo *in vitro* e *in vivo*, começando-se sempre pela avaliação da resposta inflamatória no tecido subcutâneo do animal de experimentação. Neste estudo, foram testadas três membranas de quitosano diferentes, recorrendo-se a ensaios *in vitro* e a ensaios *in vivo*, de modo a serem futuramente utilizadas como guias para a reconstrução de nervos periféricos após lesões de axonotmese e de neurotmese. As membranas de quitosano com diferentes composições foram testadas *in vitro* recorrendo-se a um sistema celular produtor de factores neurotróficos, denominado de linha celular N1E-115. Estas células N1E-115 foram cultivadas sobre os três tipos de membranas de quitosano e diferenciadas durante 48 horas, na presença de 1.5% de DMSO. A concentração intracelular de Ca^{2+} foi medida recorrendo-se à técnica de epifluorescência, nas células N1E-115 não diferenciadas e diferenciadas durante 48 horas, que foram cultivadas nas diferentes membranas de quitosano, para se avaliar a viabilidade celular na presença deste biomaterial. Os três tipos de membranas de quitosano foram testados *in vivo*, recorrendo-se ao rato como modelo animal. Para isso foi realizado implantes subcutâneos, colhidos após eutanásia dos animais para análise histológica, nas semanas 1,2, 4 e 8 após implantação. Os três tipos de membranas de quitosano são um substrato viável para a multiplicação, sobrevivência e diferenciação das células neuronais N1E-115. Além deste aspecto, os resultados obtidos na experimentação *in vivo*, demonstraram que estas membranas são candidatos promissores à reconstrução de nervos periféricos, devido à sua estrutura porosa, às modificações químicas e à grande afinidade para os sistemas celulares.

INTRODUCTION

In recent years, biomedicine suffered important advances. The use of three-dimensional materials that help tissue healing is now a current technique named guided tissue regeneration (GTR). GTR is nowadays the substitute for the traditional grafting technique as it overcomes some of its disadvantages, such as immunosuppression, limited availability of donor tissue and complications related to its sacrifice. GTR techniques are important in what concerns peripheral nerve, since many peripheral nerve injuries can only be dealt through reconstructive surgical procedures[1]. Despite continuous refinement of microsurgery techniques, peripheral nerve repair still stands as one of the most challenging tasks in neurosurgery[2-5]. Direct repair through an end-to-end suture, should be the procedure of choice whenever tension-free suturing is possible. However, patients with loss of nerve tissue resulting in a significant nerve gap, should be considered for a nerve graft procedure[6,7]. Nevertheless, this technique has some disadvantages with the most prominent being donor site morbidity, that may lead to a secondary sensory deficit and occasionally neuroma and pain. In addition, non-matching donor and inadequate recipient nerve diameters often occur, which might result in poor functional recovery[8]. Alternatives to peripheral nerve grafts include cadaver nerve segments allografts, end-to-side neurorrhaphy and entubulation by means of autologous non nervous tissues such as veins and muscles[9-13]. Experimental work from a number of laboratories have emphasized the importance of entubulation for peripheral nerve repair to manage nerve defects that cannot be bridged without tension, using completely synthetic materials that are biocompatible and biodegradable[14-16].

The concept behind entubulation is associated to the evidence that nerve cells can regenerate over a non-neuronal substrate[17]. In this promising technique, cylinder-shaped tubes are placed bridging nerve lesions, creating a microenvironment favourable for nerve regeneration, not only directing the restoring nerve fibres towards the distal nerve stump, but also allowing the incorporation of molecules and cellular systems that enhance nerve regeneration[18]. Materials used in GTR, including those used in peripheral nerve reconstruction, must comply several requisites, namely be biocompatible, non-toxic, and non-allergenic. In addition, if biomaterials are biodegradable, not only the local inflammation is reduced over the recovery period, but also, one surgical step is saved, which is a major benefit for the patient. There are many properties required for desirable nerve guided conduit and they

include permeability, that prevents fibrous scar tissue invasion but allow local revascularization to improve nutrient and oxygen supply; mechanical strength, to maintain a stable support structure for the nerve regeneration; immunological inertness with surrounding tissues; biodegradability, to prevent chronic inflammatory response or pain by nerve compression; easy regulation of conduit diameter and wall thickness, surgical facility, and so forth[17,18]. Although the availability of a wide variety of biomaterials that can replace and mimic peripheral nerve tissue whenever there is a need to overlap a considerable extension of lost tissue, the frequent occurrence of unsatisfactory recoveries dictates the development of new materials as alternatives[18].

Chitosan is a linear polysaccharide, composed of glucosamine and N-acetyl glucosamine units linked by ±(1-4) glycoside bonds derived from chitin. Depending on the source and preparation procedure, its molecular weight may range from 300 to over 1000 kD with a degree of deacetylation from 30% to 95%[19]. During the last 25 years, studies on chitosan as a biomaterial for tissue engineering applications have been intensified. This biodegradable polymer is an obvious candidate for nerve regeneration due, amidst other properties, to its anti-tumour, antibacterial and wound-healing activities[19,20]. Additionally, this biopolymer meets all the requirements for GTR: is biocompatible, non-antigenic and non-toxic[19]. Nevertheless, in order to improve both mechanical and biodegradation properties, chitosan must be cross-linked[20]. Formaldehyde, glutaraldehyde and epoxy compounds were some of the substances used for this purpose, but these cross linking agents are cytotoxic[21,22], thus limiting their use in GTR. γ-glycidoxypropyltrimethoxysilane (GPTMS) has been proved to be a good cross-linking agent combined with chitosan: the resulting hybrid membranes presented good cytocompatibility when cultured with MG63[20] and N1E-115 cells (data not published and included in this article, from our research group). Chitosan and chitosan-based materials promote adhesion, survival, and neurite outgrowth of nerve cells. Some studies have been carried out to investigate the usefulness of chitosan in nerve regeneration applications[23-27]. Researchers reported that neurons cultured on chitosan membranes can grow well and that chitosan tubes can greatly promote the repair of the peripheral nervous system[23]. Yuan et al[24], in 2004 also demonstrated that chitosan fibers supported the adhesion, migration and proliferation of Schawnn cells (SCs), providing a similar guide for regenerating axons to Büngner bands in the nervous system[24,25]. In another study, Itoh et al in 2003, prepared hydroxyapatite-coated chitosan tubes, including laminin-1 or laminin peptides as scaffolds for peripheral nerve reconstruction[26]. These tubes improved the growth of regenerating axons bridging a 15mm defect in the sciatic nerve. Cao et al, in 2005 studied the physical, mechanical and degradation properties of chitosan films and the affinity between SCs and the films[27]. Three kinds of cross-linked chitosan films were prepared with hexamethylene diisocyanate, epichlorohydrin and glutaraldehyde. Cross linking decreased the swelling degree and the degradation rate of the chitosan membranes, whereas it increased their hydrophilicity and elastic modulus, and also enhanced the spread and proliferation of SCs[27].

The three chitosan membranes, developed by our research group and presented in this study, were tested *in vitro* and *in vivo*, to demonstrate both their cytocompatibility and biocompatibility for posterior use as nerve tube-guides for the reconstruction of rat sciatic nerves submitted to lesions of axonotmesis or neurotmesis. These studies in accordance to the available bibliography clearly suggest that chitin and chitosan support nerve cell adhesion and neurite outgrowth, making these materials potential candidates for scaffolds in neural tissue engineering.

MATERIALS AND METHODS

Membranes Preparation

Chitosan (high molecular weight, Aldrich®, USA) was dissolved in 0.25M acetic acid aqueous solution to attain a concentration of 2% (w/v). To obtain type II and type III membranes, GPTMS (Aldrich®, USA) was also added to the chitosan solution and stirred at room temperature for 1h to obtain type II and type III membranes. The solutions for type I and II chitosan membranes were then poured into covered polypropylene containers, and aged at 60°C for 2 days. The aging process for type III chitosan membranes was significantly different: membranes were frozen for 24h at -20ºC and then transferred to the freeze drier, where they were left for 12h to complete dryness. The chitosan membranes (types I, II and III) were soaked in 0.25N sodium hydroxide to neutralize remaining acetic acid, washed well with distilled water, and dried at 37°C for 1 day (types I and II) or freeze dried (type III). All membranes were sterilized with ethylene oxide gas (EO), considered by some authors the most suitable method of sterilization for chitosan membranes[28]. Prior to their use *in vivo*, membranes were kept during 1 week at room temperature in order to loose any EO remnants.

***In vitro* testing: N1E-115 cell culture and $[Ca^{2+}]_i$ measurements**

N1E-115 cell line is a clone derived from mouse neuroblastoma C-1300, and preserves several important properties from differentiated neuronal cells in culture, namely biochemical, physiological and morphological[29]. This cellular system undergoes neuronal differentiation when cultured in the presence of dimethylsulfoxide (DMSO), adenosine 3'5'-cyclic monophosphate (cAMP), or serum withdrawal[29], exhibiting characteristics from neural cells, such as ceased multiplication, extensive neurite outgrowth and polarization of cellular membranes, being able to locally produce and deliver nerve growth factors when used to reconstruct peripheral nerve lesions[29].

In neuronal cells the regulation of the intracellular free calcium concentration $[Ca^{2+}]_i$ plays an important role in physiological processes such as growth and differentiation, controlling important cell functions like the release of neurotransmitters and the membrane's excitability. The mechanisms that control $[Ca^{2+}]_i$ are of crucial importance for normal homeostasis, and its deregulation has been associated to cellular changes and even cell death[30], when $[Ca^{2+}]_i$ reach values above 105 nM[29]. N1E-115 cells were cultured over 2cm x 2cm chitosan membrane fragments (type I, type II, and type III), attached to poly-l-lysine coated glass coverslips in Petri dishes (around 2 x 10^6 cells/Petri dish), at 37ºC, 5% CO_2 in an humidified atmosphere (Nuaire). Maintenance medium consisted in 89.8% Dulbecco's Modified Eagle's Medium (DMEM + GlutaMAX; Gibco), supplemented with 10% foetal bovine serum (FBS; Sigma), 0.1% penicillin (10.000 U/ml)/streptomycin (10 mg/ml) (Sigma) and 0.1% amphotericin B (250µg/ml, Sigma). The culture medium was changed every 48h, and cell culture was observed daily in an inverted microscope (Zeiss, Germany). After reaching 80% of cellular confluence, N1E-115 cells were supplied with differentiation medium (95.8% DMEM + GlutaMAX, 2.5% FBS, 0.1% penicillin/streptomycin, 0.1% amphotericin B and 1.5% DMSO (Sigma). The $[Ca^{2+}]_i$ was determined in N1E-115 cell culture before differentiation and 48h after its transfer to the differentiation medium containing 1.5% DMSO. $[Ca^{2+}]_i$ was measured in Fura-2-AM-loaded cells using dual wavelength spectrofluorometry as previously described[29] and here summarized. The common fluorescent indicators for Ca^{2+} are polcarboxylate anions that cannot cross lipid bilayer membranes and therefore are not cell permeable. By far the most convenient way of loading an indicator into cells is incubating the cells in a dilute solution of the AM ester of the indicator[31]. N1E-115 mouse neuroblastoma cells were loaded with Ca^{2+} indicator by incubation in 2.5mM Fura-2 acetixymethyl ester (Fura-2-AM, Molecular Probes) and 0.03% Pluronic (Molecular Probe) in a Ringer Solution with the following composition: 121 mM NaCl, 5.4 mM KCl, 9 mM D-glucose, 1.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 6 mM $NaHCO_3$, and 25 mM HEPES, with a pH of 7.4; at 37ºC in darkness for 60 minutes. After loading Fura-2-AM, N1E-115 cells were washed in Ringer Solution (121 mM NaCl, 5.4 mM KCl, 9 mM D-glucose, 1.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 6 mM $NaHCO_3$, and 25 mM HEPES, with a pH 7.4). The AM ester form of the indicator is uncharged and hydrophobic. On the other hand, the indicator carboxyl groups are essential for its ability to sense Ca^{2+}. Therefore, the AM group is labile to enzymatic hydrolysis by cellular estereases. The glass cover slips with the adhering N1E-115 cells to the chitosan membranes were transferred to a glass chamber containing 100µl of the Ringer Solution. The chamber was placed in a well on the stage of an epifluorescence microscope (Zeiss, Germany). Fluorescence measurements were performed in each individual cell. The emitted fluorescence intensities at 510 nm were acquired by computer software, which registered the number of photons emitted per second, during 30s for each 340 nm and 380 nm excitation wavelengths. The $[Ca^{2+}]_i$ was estimated from the ratio equation described by Grynkiewicz and colleagues[31]. For determination background fluorescence, cells were incubated in 2.5 mM 4-br-A23186 (Molecular Probe) and 10 mM $MnCl_2$ in 100 µl of Ringer solution at room temperature in darkness for 10 minutes. The $[Ca^{2+}]_i$ measurements considered for these results were the ones which the background signal was inferior to 20% of the total emitted fluorescence.

***In vivo* assay in a rat model**

The experimental group consisted of 4 Wistar female rats, weighting between 250-350g. Two animals were housed per cage (Makrolon type 4, Tecniplast, VA, Italy), in a temperature and humidity controlled room with 12-12h light/dark cycles, and were allowed normal cage activities under standard laboratory conditions. The animals were fed with standard chow and water *ad libitum*. Adequate measures were taken to minimize pain and discomfort taking into account human endpoints for animal suffering and distress. All procedures were performed with the approval of the Veterinarian Authorities of Portugal, and in accordance with the European Communities Council Directive of November 24th 1986 (86/609/EEC).

Anaesthesia was achieved with an intraperitoneal (IP) injection of a pre-mixed solution consisting in ketamine (Imalgène 1000®), 100 mg/kg body weight (bw), and xylazyne (Rompun®), 200 mg/kg bw. Hair from the dorsal area was

Fig. 1 – In vivo *experimental group, the rat was the animal model used. Chitosan membranes were implanted subcutaneously in fragments of 2cmx2cm: left-cranial incision for type I chitosan membrane (incision 1), mid-right and left-caudal incisions for type II (incision 2) and type III chitosan membrane (incision 3), respectively.*

clipped and the skin scrubbed in a routine fashion with an iodopovodone 10% solution (Betadine®). Three 1.5-2cm long linear incisions, were made (incision 1 = left-cranial; incision 2 = mid-right; incision 3 = left-caudal). After blunt dissection towards the ventral aspect of the body, the membrane fragments, 2cmx2cm, were implanted subcutaneously: type I chitosan in incision 1, type II chitosan in incision 2 and type III chitosan in incision 3 (Figure 1). Skin and subcutaneous tissues were closed with a simple-interrupted suture of a non-absorbable filament (Synthofil®, Ethicon). An antibiotic (enrofloxacin, Alsir® 2.5%, 5mg/kg b.w., subcutaneously) was administered to prevent any infections. On days 7, 14, 30 and 60, one animal from each group was randomly selected; after performing the same anaesthetic protocol, skin and subcutaneous tissues from the implant area were collected and fixed in a container with 10% formaldehyde solution for posterior histological evaluation. The rats were then euthanized, by lethal intracardiac injection of 5% sodium pentobarbital (Euthasil®).

Samples were routinely processed, and 5 µm-thin sequential sections were stained with hematoxilin-eosin (HE) and Masson's trichrome stain for accurate observation of the fibrotic reaction surrounding the three types of implants tested *in vivo*. Collagen stains blue with this technique. The thickness of the fibrous capsules was assessed as the mean of three similar measurements, obtained from representative sections, using a DS-5Mc digital camera (Nikon). All histological observations were made on an E600 light microscope (Nikon).

SEM analysis

The surface morphology of membranes was observed under a scanning electron microscope (SEM; JEOL JSM 6301F) equipped with X-ray energy dispersive spectroscopy (EDX) microanalysis capability, (Voyager XRMA System, Noran Instruments).

Statistic analysis

All data were presented as mean ± SEM, where N is the number of cells where the $[Ca^{2+}]_i$ was measured by the epifluorescence technique or N is the number of animals tested per each histological evaluation or measurement. All statistical tests were Student's t test[32]. The given P values correspond to errors of the second kind ($P < 0.05$)[32].

	Type I chitosan membranes	Type II chitosan membranes	Type III chitosan membranes
Non-differentiated N1E-115 cell line	39,9 ± 3,4 N = 15	35,9 ± 3,2 N = 15	40,2 ± 2,9 N = 15
Differentiated N1E-115 cell line	42,9 ± 5,1 N = 15	44,3 ± 4,8 N = 15	41,6 ± 4,3 N = 15

Table 1 – *$[Ca^{2+}]_i$ measurements in N1E-115 cells obtained by the epifluorescence technique, using Fura-2-AM probe, before differentiation and 48h after differentiation in the presence of 1.5% DMSO. Values are presented as mean ± standard error of the mean (SEM). N corresponds to the number of individual N1E-115 cells analysed. All statistical tests were Student's t test.*

RESULTS AND DISCUSSION

$[Ca^{2+}]_i$ determination in N1E-115 cell line

Results obtained from epifluorescence technique are presented in Table 1, and are referred to measurements from non-differentiated N1E-115 cells and after 48h of differentiation in the presence of 1.5% DMSO. The mean value of $[Ca^{2+}]_i$ in non-differentiated N1E-115 cells (N = number of cells submitted to $[Ca^{2+}]_i$ measurement) was 39.9 ± 3.4 nM (N = 15), 35.9 ± 3.2 nM (N = 15) and 40.2 ± 2.9 nM (N = 15), for cultures over chitosan membranes, type I,II and III, respectively. Values of $[Ca^{2+}]_i$ for N1E-115 cells after 48h of differentiation in the presence of 1.5% DMSO were 42.9 ± 5.1 nM (N = 15), 44.3 ± 4.8 nM (N = 15) and 41.6 ± 4.3 nM (N = 15), for cultures over chitosan membranes, type I,II and III, respectively. All these values are not statistically different for $P < 0.05$, and correspond to $[Ca^{2+}]_i$ from cells that did not begin the apoptosis process although the evident neural differentiation. According to this fact, it is reasonable to conclude that chitosan membranes, previously presented as type I, II and III, were a viable substrate for N1E-115 neuronal cell line adhesion, multiplication and differentiation.

In vivo assay in a rat model

Figure 2 (Fig. 2A and Fig. 2B) shows the SEM microstructure of type II and type III membranes, respectively. Wettability of material surfaces is one of the key factors for protein adsorption, cell attachment and migration[33]. The addition of GPTMS improved the wettability of chitosan surfaces as shown in a previous paper[20], and therefore membranes type II and III are more hydrophilic when compared to the membrane type I. As Tateishi et al pointed out[34], the scaffolds should not only promote cell adhesion, cell proliferation, and cell differentiation, but also be biocompatible, biodegradable, highly porous with a large surface to volume ratio, mechanically strong enough for handling, and capable of being formed into desired shapes. So, in this work different approaches were followed to produce porous and non-porous membranes. The drying techniques employed to prepare the membranes were freeze-drying (type III) and the conventional thermal drying (type I and II), which led to extremely dissimilar microstructural (Figure 2) and mechanical properties as previously reported[17,18]. The former technique, which consists of a rapid solidification of the solvent, obtained by lowering the temperature, followed by sublimation under vacuum led to a 3D porous microstructure. The lyophilized porous chitosan-GPTMS hybrids (type III) showed sponge-like elasticity, compared to the stiffer chitosan membrane (type I). Previous studies

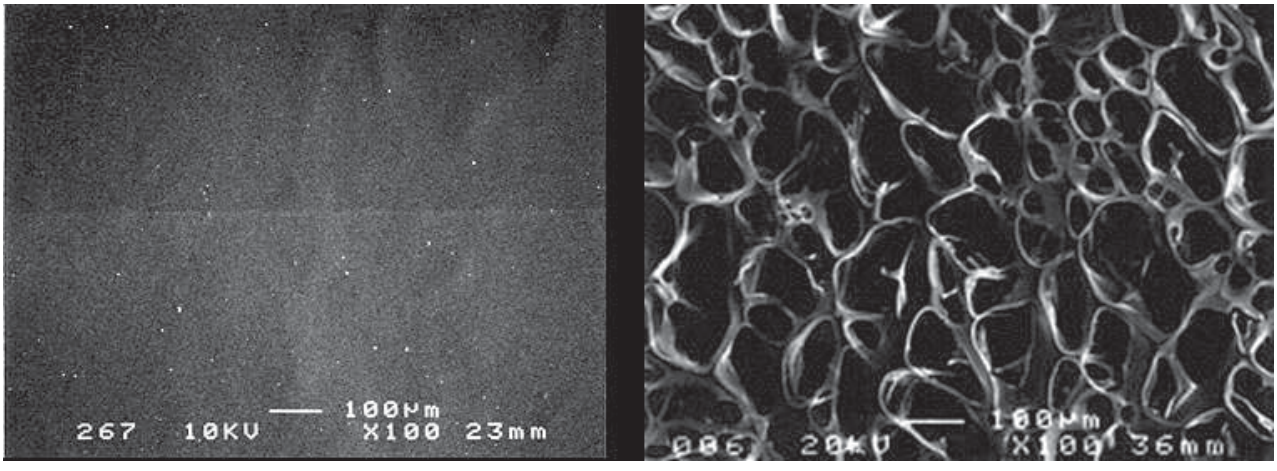

Fig. 2 – *SEM microstructure of chitosan membranes. A. Type II chitosan membrane. B. Type III chitosan membrane, showing a more porous microstructure, when compared to Type II chitosan membrane.*

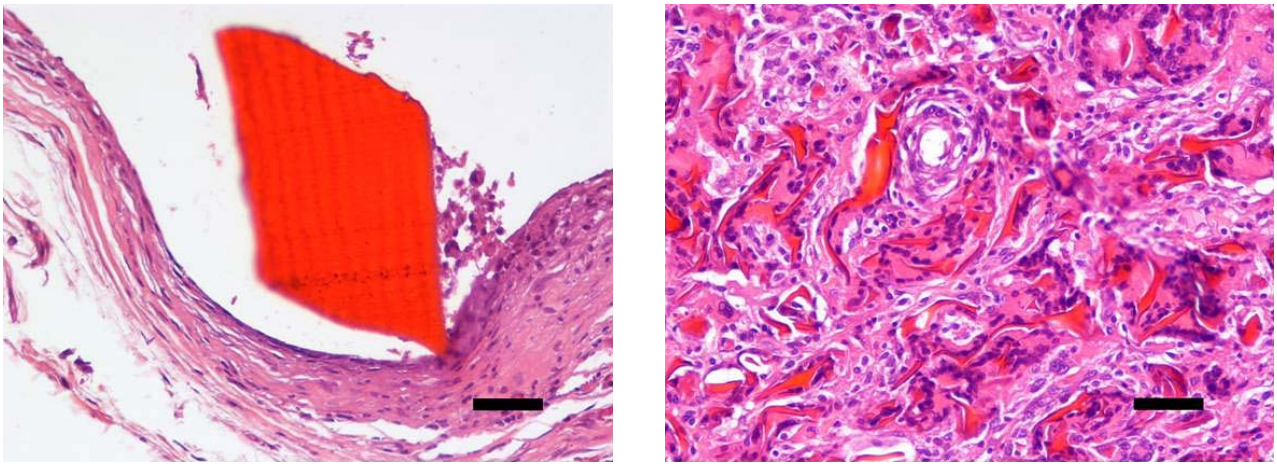

Figure 3 – *Images from type II and III chitosan membranes (Figure 3A and Figure 3B, respectively), retrieved on week-8. Samples were stained with hematoxylin-eosin. Magnification of 200x. Bar = 50 µm. Red fragments correspond to chitosan membrane. Note moderate fibrous capsule on Figure 3A and granulomatous reaction on Figure 3B.*

reported that the pore size of the structure of the hybrid membranes is controllable through the freezing temperature, that is, the higher freezing temperature yields the larger pores and almost not affected by the GPTMS content, but GPTMS content affects the porosity level. In the study, membrane type III has about 110µm pores and 90% of porosity as reported elsewhere[35].

Images shown in Figure 3 correspond to type II and III chitosan membranes (Fig. 3A and Fig. 3B, respectively), retrieved on week-8. These samples were stained with hematoxylin-eosin (HE). The red fragments observed, correspond to the chitosan membranes. A moderate fibrous capsule can be observed on Figure 3A and a granulomatous reaction on Fig. 3B.

Images shown in Figure 4 (Fig. 4A, Fig. 4B and Fig. 4C) and Figure 5 (Fig. 5A, Fig. 5B and Fig. 5C) were obtained from samples retrieved on week-2 and week-8, from type I, II and III chitosan membranes, respectively. On a preliminary histological analysis, chitosan membranes elicited a chronic inflammatory reaction on the implantation site, even on samples retrieved as early as 7 days, with an increasing gradient from type I to type III chitosan membranes. Whereas types I and II elicited mild chronic inflammation, characterized by infiltration of small numbers of macrophages, lymphocytes and plasma cells (Fig. 4A and Fig. 5A for type I chitosan membranes; Fig. 4B and Fig. 5B for type II chitosan membranes), type III chitosan membranes induced a strong granulomatous reaction, with abundant multinucleated giant cells (Fig. 4C and Fig. 5C). Mild peripheral fibrosis in the form of a collagen capsule was observed with types I and II chitosan membranes, while type III induced significant interstitial fibrosis. On week-2, capsule thickness was 23.98 µm, 24.71 µm and 24.71 µm (mean value of 24.47± 0,24µm, N = 4) for type I

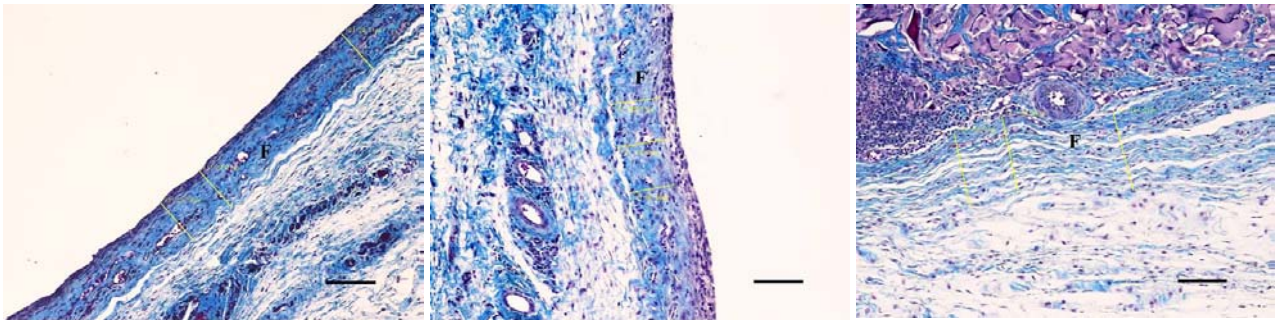

Fig. 4 – *Images from samples retrieved on week-2 and obtained under light microscope, after standard histological processing and staining with Masson's trichrome. Magnification of 100x. Bar = 100 µm. Thin yellow parallel lines indicate 3 representative measurements of the capsule's thickness. The blank spaces correspond to areas previously occupied by the membranes, which were lost during histological processing.* ***A.*** *Type I chitosan membrane: there is a thin fibrous capsule (**F**) and a mild mononuclear inflammatory reaction.* ***B.*** *Type II chitosan membrane: there is a mild fibrous capsule (**F**) and a discrete mononuclear inflammatory reaction.* ***C.*** *Type III chitosan membrane: granulomatous reaction surrounding small membrane fragments (red) with interstitial fibrosis and a thick fibrous capsule (**F**).*

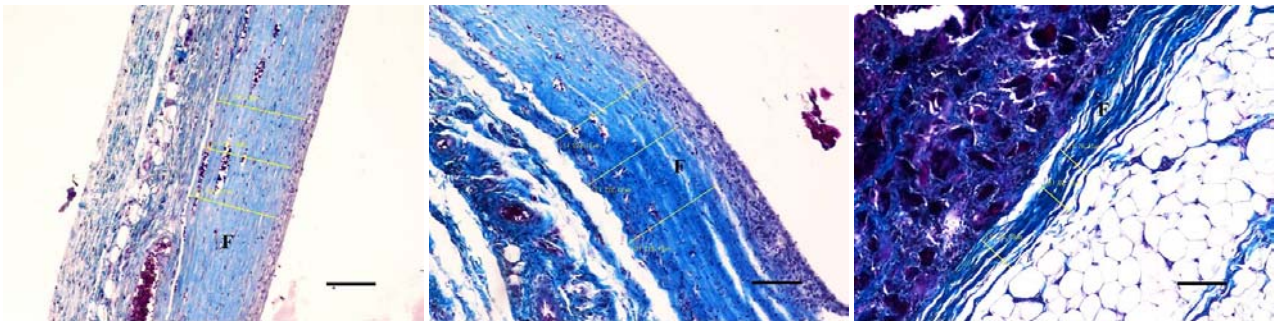

Fig. 5 – Images from samples retrieved on week 8 and obtained under light microscope, after standard histological processing and staining with Masson´s trichrome. Magnification of 100x. Bar = 100 ¼m. Thin yellow parallel lines indicate 3 representative measurements of the capsule's thickness. The blank spaces correspond to areas previously occupied by the membranes, which were lost during processing. A. Type I chitosan membrane: note the increased capsule thickness (F) compared with Figure 4A. B. Type II chitosan membrane: note the increased capsule thickness (F) compared with Figure 4B. C. Type III chitosan membrane: granulomatous reaction surrounding small membrane fragments (dark red) with interstitial and peripheral fibrosis (F) in blue.

chitosan membranes (Fig. 4A). Measurements were 102.57 µm, 99.62 µm and 95.45 µm (mean value of 99.21 ± 2.07µm, N = 4) for chitosan type II membrane (Fig. 4B) and 155.87 µm, 159.35 µm, 164.21 µm (mean value of 159.81 ± 2.42 µm, N = 4) for type III chitosan membrane (Fig. 4C). On week-8, measurements were 190.21 µm, 185.16 µm, 185.57 µm (mean value of 186.98 ± 1.62µm, N = 4) for type I chitosan membrane (Fig. 5A); 224.16 µm, 222.06 µm, 219.45 µm (mean value of 221.89 ± 1.36µm, N = 4) for type II chitosan membrane (Fig. 5B) and 84.89 µm, 82.16 µm, 78.41 µm (mean value of 81.82 ± 1.88µm, N = 4) for type III chitosan membrane (Fig. 5C). Although, type III chitosan membranes presented the stronger inflammatory reaction on week-4, both with a strong cellular component and a thick capsule, smaller values for capsule thickness were obtained on week-8. None of the tested membranes was rejected throughout the 60-day healing period, nor elicited systemic or local clinical signs of illness, infection or inflammation, with all animals remaining healthy throughout the study period.

Interestingly and opposing to what was observed in the rat model, a similar study in sheep performed by our research team[36] demonstrated that the subcutaneously implanted hybrid chitosan membranes seemed to induce a stronger inflammatory reaction, not only with a thicker collagen capsule, but also with higher density cellular inflammatory infiltrate. These results might be explained by the fact that the immunological system response is distinct from that of the rat. On the other hand, the three types of chitosan membranes tested in sheep did not induce local or systemic clinical symptoms of rejection[36].

Chitosan matrices have been shown to have low mechanical strength under physiological conditions and to be unable to maintain a predefined shape for transplantation, which has limited their use as nerve guidance conduits in clinical applications. The improvement of their mechanical properties can be achieved by modifying chitosan with a silane agent. GPTMS is one of the silane-coupling agents, which has epoxy and methoxysilane groups. The epoxy group reacts with the amino groups of chitosan molecules, while the methoxysilane groups are hydrolyzed and form silanol groups, and the silanol groups are subjected to the construction of a siloxane network due to condensation. Thus, the mechanical strength of chitosan networks can be improved by cross linking chitosan with GPTMS.

In the present study, despite the same composition, type II and type III membranes presented a distinct behaviour: the latter elicited an exuberant cellular infiltrate composed in a large extent by multinucleated giant cells and some mast cells, whereas type II chitosan elicited a mild fibrous capsule and a discrete inflammatory reaction. Type III chitosan membranes underwent a completely different aging process, the so called freeze drying. This lyophilisation procedure resulted in highly porous membranes: after freezing and lyophilisation, the spaces formerly occupied by the solvent were left emptied so these porous membranes presented a superior surface/volume ratio, when compared to the type I and type II chitosan membranes[37]. As surface/volume ratio increases, there is a higher contact surface with the host's immune system, which could explain the resulting exuberant cellular component. Nevertheless, additional studies are needed to completely understand the factors that led to these differences in the host's inflammatory response.

Tissue engineering termed as Regenerative Medicine is regarded as an ultimately ideal medical treatment for repairing tissues, which include the peripheral nerve. This biomedical engineering employs three fundamental tools: living cells, signal molecules, and scaffolds. Chitosan is

one of the most promising biomaterials in tissue engineering as it offers a distinct set of advantageous physico-chemical and biological properties. In fact, this study demonstrated that the three chitosan membranes tested *in vitro* and *in vivo* were biocompatible and therefore an important scaffold for the reconstruction of peripheral nerve, after axonotmesis or neurotmesis injury, associated or not to neurotrophic factors cellular producing systems[38]. This study, in accordance to the available bibliography, clearly suggests that GPTMS hybrid membranes provide a suitable scaffolding environment for neural tissue engineering, making this material a potential candidate for scaffolds in neural tissue engineering.

ACKNOWLEDGEMENTS

The authors would like to gratefully acknowledge the valuable support by José Manuel Correia Costa, from Laboratório de Parasitologia, Instituto Nacional de Saúde Dr. Ricardo Jorge, Porto, Portugal, and Paulo Ferreira from Centro de Estudos de Ciência Animal (CECA) do Instituto de Ciências e Tecnologias Agrárias e Agro-Alimentares (ICETA) from University of Porto, Portugal.

Conflito de interesses:
Os autores declaram não ter nenhum conflito de interesses relativamente ao presente artigo.

Fontes de financiamento:
This work was supported by the Fundação para a Ciência e Tecnologia (FCT) through the financing of the research project PTDC/CVT/64220/2006

REFERENCES

1. KLINE DG: Spinal nerve root repair after brachial plexus injury. J Neurosurg 2000;93:336-8
2. MADISON RD, ARCHIBALD SJ, KRARUP C: Peripheral nerve injury. I.K. Cohen, F. Diegelman and W.J. Lindbald, Philadelphia 1992;pp.450-487
3. HOKE A: Mechanisms of Disease: what factors limit the success of peripheral nerve regeneration in humans? Nat Clin Pract Neurol 2006;2:448-454
4. LUNDBORG G: Enhancing posttraumatic nerve regeneration. J Peripher Nerv Syst 2002;7:139-140
5. MILLESI H: Progress in peripheral nerve reconstruction. World J Surg 1990; 14:733-747
6. LUNDBORG G: Bridging nerve defects: The role of tissue interpositioning. A.C. Masquelet and A.C. Ferreira, London 2003; pp.153-167
7. MATSUYAMA T, MACKAY M, MIDHA R: Peripheral nerve repair and grafting techniques: a review. Neurol Med Chir (Tokyo) 2000;40:187-199
8. MAY M: Trauma to the facial nerve. Otolaryngol Clin North Am 1983;16:661-670
9. DOOLABH VB, HERTL MC, MACKINNON SE: The role of conduits in nerve repair: a review. Rev Neurosci 1996;7:47-84
10. JENSEN JN, TUNG TH, MACKINNON SE, BRENNER MJ, HUNTER DA: Use of anti-CD40 ligand monoclonal antibody as antirejection therapy in a murine peripheral nerve allograft model. Microsurgery 2004;24:309-315
11. LUNDBORG G: Alternatives to autologous nerve grafts. Handchir Mikrochir Plast Chir 2004;36:1-7
12. GEUNA S, PAPALIA I, TOS P: End-to-side (terminolateral) nerve regeneration: a challenge for neuroscientists coming from an intriguing nerve repair concept. Brain Res Rev 2006;52:381-8
13. CHEN MB, ZHANG F, LINEAWEAVER WC: Luminal fillers in nerve conduits for peripheral nerve repair. Ann Plast Surg 2006;57:462-471
14. DEN DUNNEN WF, ROBINSON PH, VAN WESSEL R, PENNINGS AJ,. VAN LEEUWEN MB, SCHAKENRAAD JM: Long-term evaluation of degradation and foreign-body reaction of subcutaneously implanted poly(DL-lactide-epsilon-caprolactone). J Biomed Mater Res 1997;36:337-346
15. SCHMIDT CE, LEACH JB: Neural tissue engineering: strategies for repair and regeneration. Annu Rev Biomed Eng 2003;5:293-347
16. BATTISTON B, GEUNA S, FERRERO M, TOS P: Nerve repair by means of tubulization: literature review and personal clinical experience comparing biological and synthetic conduits for sensory nerve repair. Microsurgery 2005;25:258-267
17. SUEMATSU N: Tubulation for peripheral nerve gap: its history and possibility. Microsurgery 1989;10:71-4.
18. LUIS AL, RODRIGUES JM, AMADO S et al: PLGA 90/10 and caprolactone biodegradable nerve guides for the reconstruction of the rat sciatic nerve. Microsurgery 2007;27:125-137
19. Chandy T, Sharma CP: Chitosan – as a biomaterial. Biomater Artif Cells Artif Organs 1990;18:1-24
20. SHIROSAKI Y, TSURU K, HAYAKAWA S et al: In vitro cytocompatibility of MG63 cells on chitosan-organosiloxane hybrid membranes. Biomaterials 2005;26:485-493
21. CHVAPIL M, SPEER D, MORA W, ESKELSON C: Effect of tanning agent on tissue reaction to tissue implanted collagen sponge. J Surg Res 1983;35:402-9
22. NISHI C, NAKAJIMA N, IKADA Y: In vitro evaluation of cytotoxicity of diepoxy compounds used for biomaterial modification. J Biomed Mater Res 1995;29:829-834
23. HAIPENG G, YINGHUI Z, JIANCHUN L, YANDAO G,. NANMING Z, XIUFANG Z: Studies on nerve cell affinity of chitosan-derived materials. J Biomed Mater Res 2000;52:285-295
24. YUAN Y, ZHANG P, YANG Y, WANG X, GU X: The interaction of Schwann cells with chitosan membranes and fibers in vitro. Biomaterials 2004;25:4273-8
25. BUNGE RP: The role of the Schwann cell in trophic support and re generation. J Neurol 1994;242:S19-21
26. ITOH S, YAMAGUCHI I, SUZUKI M et al: Hydroxyapatite-coated tendon chitosan tubes with adsorbed laminin peptides facilitate nerve regeneration in vivo. Brain Res 993 (2003), 111-23.
27. CAO W, CHENG M, AO Q, GONG Y, ZHAO N, ZHANG X: Physical, mechanical and degradation properties, and schwann cell affinity of cross-linked chitosan films. J Biomater Sci Polym Ed 2005;16:791-807
28. MARRECO PR, DA LUZ MOREIRA P, GENARI SC, MORAES AM: Effects of different sterilization methods on the morphology, mechanical properties, and cytotoxicity of chitosan mem-

branes used as wound dressings. J Biomed Mater Res B Appl Biomater 2004;71:268-277
29. RODRIGUES JM, LUÍS AL, LOBATO JV et al: Determination of the intracellular Ca^{2+} concentration in the N1E-115 neuronal cell line in perspective of its use for peripheric nerve regeneration. Biomed Mater Eng 2005;15:455-465
30. TRUMP BF, BEREZESKY IK: Calcium-mediated cell injury and cell death. Faseb J 1995;9:219-228
31. GRYNKIEWICZ G, POENIE M, TSIEN RY: A new generation of Ca2+ indicators with greatly improved fluorescence properties. J Biol Chem 1985;260:3440-50
32. HALD A, WILEY J: New York 1952;pp.391-4
33. HENCH LL, ETHRIDGE EC: Biomaterials An Interfacial Approach. A. Press, New York 1982;pp.10
34. TATEISHI T., CHEN G, USHIDA T: Biodegradable porous scaffolds for tissue engineering. J Artif Organs 2002;5:77-83
35. SHIROSAKI Y, OKAYAMA T, TSURU K, HAYAKAWA S, OSAKA A: Synthesis and cytocompatibility of porous chitosan-silicate hybrids for tissue engineering scaffold application. Chemical Engineering Journal (submitted).
36. CORTEZ PP, SHIROSAKI Y, BOTELHO CM: Hybrid chitosan membranes tested in sheep for guided tissue regeneration. Key Engineering Materials 2008;361-363:1265-8
37. SHIROSAKY Y: Synthesis of two- and three- dimensional organic-inorganic hybrids for inducing biomineralization, Ph.D. Dissertation, The Graduate School of Natural Science and Technology, Okayama University 2005
38. SIMÕES MJ, AMADO S, GEUNA S et al: Functional and Morphological Assessment of a Standardized Rat Sciatic Nerve Crush Injury with a Non-Serrated Clamp Reconstructed with Chitosan and Collagen Membranes Associated to a Cellular System (Poster). in 10th Meetting Portuguese Society for Neurosciences. Ofir, Esposende. Portugal 2007.