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**Abstract**

The current trend of peripheral nerve tissue engineering is the design of advanced nerve guidance channels (NGCs) acting as physical guidance for regeneration of nerves across lesions. NGCs should present multifunctional properties aiming to direct the sprouting of axons from the proximal nerve end, to concentrate growth factors secreted by the injured nerve ends, and to reduce the ingrowth of scar tissue into the injury site. A critical aspect in the design of NGCs is conferring them the ability to provide topographic, chemotactic and haptotactic cues that lead to functional nerve regeneration thus increasing the axon growth rate and avoiding or minimizing end-organ (e.g. muscle) atrophy. The present work reviews the recent state of the art in NGCs engineering and defines the external guide and internal fillers structural and compositional requirements that should be satisfied to improve nerve regeneration, especially in the case of large gaps (> 2cm). Techniques for NGCs fabrication were described highlighting the innovative approaches direct to enhance the regeneration of axon stumps compared to current clinical treatments. Furthermore, the possibility to apply stem cells as internal cues to the NGCs was discussed focusing on scaffold properties necessary to ensure cell survival. Finally, the optimized features for NGCs design were summarized showing as multifunctional cues are needed to produce NGCs having improved results in clinics.

**Table of contents**

## 1. Introduction

*1.1 Nerve injury*

Peripheral nerves are commonly exposed to physical injuries, usually caused by transportation and construction accidents, natural disaster and war damage, and other traumas, including iatrogenic side effects of surgery. Peripheral nerve injuries affect 2.8% of trauma patients, frequently leading to life-long disability (Wiberg and Terenghi, 2003). The incidence of nerve injuries is relatively high all over the world. In US over 200,000 peripheral nerve repair procedures are performed annually (Ichihara et al., 2008). Around 5% of wounds in the extremities can be associated with peripheral nerve injuries (Huang and Huang, 2006). In contrast with central nervous system (CNS), peripheral nervous system (PNS) has the intrinsic capacity to regenerate at a certain extent after injury (Meek et al., 2002; Schmidt and Leach, 2003).

Nerve injuries were classified by Sunderland in five grades depending on the severity degree (Sunderland, 1951). Neurapraxia (grade 1) is related to a block in the fibre conduction; axonotmesis (grade 2) is associated to axon transection with an intact endoneurium; neurotmesis/neurotmesis +/ neurotmesis ++ (grade 3/ grade 4/ grade 5) range from transaction with intact perineurium to a completely interrupted nerve. Spontaneous regeneration is influenced by the severity of the injury and occurs in axonotmesis with a regeneration rate of 1–3 mm/day, resulting in functional recovery within few weeks for injury around 1 cm. In case of severe injuries, when the nerve trunk has been completely interrupted (neurotmesis) and the missing gap is large (higher than 2 cm) the spontaneous regeneration cannot be achieved.

*1.2 Nerve regeneration process*

The regeneration process starts immediately after injury and is based on three different phases (Deumens et al., 2010). The early phase (1-5 days) is characterized by axon and myelin degeneration that occurs at distal and proximal sites (Wallerian degeneration). At the same time, the nucleus of injured neurons adopts an eccentric position within the cell body and the nucleolus becomes more prominent; neuronal biochemistry and function are altered with an increase of protein synthesis required for axonal sprouting and growth. During the intermediate phase (from 5 days to weeks), macrophages infiltrate at the injured site contributing to cellular and tissue debris removal, Schwann cells (SCs) start a robust proliferation due to the lost contact with axons and proximal stumps develop regenerative axonal sprouts. The newly generated SCs, together with pre-existing SCs that survived the nerve injury, form the bands of Büngner. The bands of Büngner in the distal nerve segment are highly aligned tubes formed by basal lamina secreted by SCs. The topographical property of these bands is crucial for the directional guidance of axon growth, as the growth cones of sprouting axons use bands of Büngner as a regenerative substrate. In the last phase of the regeneration process (from weeks to months), the growth cone of sprouting axons extend within bands of Büngner at a rate of 1-3 mm/day resulting in complete axon regeneration and functional recovery.

The described spontaneous regeneration process occurs in axonotmesis while can be compromised in neurotmesis where endoneurial tubes are damaged. In these cases, SCs and fibroblasts proliferate and re-organize in the attempt to re-establish a connective bridge across the lesion, while distal stumps release chemotactic cues to attract axon sprouts. The spontaneous regeneration process often fails resulting in abnormal sprouting and neuroma formation. In neurotmesis, reconstructive surgery is required to achieve anatomical and functional regeneration, exploiting the knowledge of clinical and translational neurosciences (Wiberg and Terenghi, 2003).

*1.3 Current clinical approaches for treating peripheral nerve injuries*

End-to-end suturing (nerve coaptation) is the preferred strategy for peripheral nerve regeneration (A.B. Sanghvi, 2004) and can be applied to repair short nerve gaps. However, when nerve gap is large and end-to-end reconnection of transected nerve stumps cannot be performed without exerting tension, a bridging nerve device is necessary (Ichihara et al., 2009). Autograft, i.e. the implantation of a portion of an autologous nerve, is the preferred clinical strategy for the treatment of peripheral nerve injuries, as the biomimetic properties of the implanted conduit favor regeneration (Dellon and Mackinnon, 1988). Drawbacks of this clinical approach include: the possible mismatch between the diameter of the injured nerve and the autograft, the lack of an adequate amount of autologous nerve tissue for implantation, neuroma formation at the donor site and the necessity of at least two surgeries at injured site and at donor site with consequent donor site morbidity and relevant sanitary costs (Schmidt and Leach, 2003). Beside the above disadvantages, only 40-50% of patients receiving autografts achieve a successful degree of functional recovery (Lee and Wolfe, 2000). For this reason, new alternative strategies for peripheral nerve regeneration are under study and development.

Muscle-in-vein conduits have been proposed as an efficient strategy for peripheral nerve regeneration, making use of autologous tissues (Battiston et al., 2009; Brunelli et al., 1993). The technique consists of the implantation of a guide based on a vein, filled with skeletal muscle tissue providing haptotactic and chemotactic cues to the regenerating axons. Although surgeons can apply this treatment by a single surgery, drawbacks are associated with the use of autologous tissue, including the healing from multiple injuries and the lack of an adequate amount of autologous tissue for the treatment of severe nerve injuries.

Concerning artificial grafts, several bioresorbable NGCs have been approved by US Food and Drug Administration for human uses, based on type I collagen (Neuragen®, Neuroflex™, NeuroMatrix™, NeuraWrap™, NeuroMend™), porcine small intestinal submucosa (Surgis® Nerve Cuff), poly(glycolic acid) (Neurotube®) and poly(D,L lactide-co-ε-caprolactone)

(Neurolac®). However, they are not recommended for larger gaps than 3 cm (Kehoe et al., 2012) and their biological performances are inferior to autograft that remains the gold standard treatment for bridging approaches.

*1.4 Tissue Engineering strategies for peripheral nerve regeneration*

Large gaps need alternative clinical solutions to autologous approaches, improving recovery rates and functional outcome. Current research is focused on the development of engineered "nerve guides" or "nerve guidance channels" (NGCs) that physically guide regeneration of nerves across lesions, directing the sprouting of axons from the proximal nerve end, concentrating growth factors secreted by the injured nerve ends, and reducing the ingrowth of scar tissue into the injury site (Taras and Jacoby, 2008). Up to now, a wide range of natural or synthetic biomaterials, together with well-defined fabrication techniques, have been used to prepare NGCs with different structural and physicochemical properties (Chiono et al., 2009a; Ciardelli and Chiono, 2006; Johnson and Soucacos, 2008).

Biological and physicochemical requirements, such as biocompatibility, biodegradability, adequate mechanical properties and permeability may be achieved by properly tailoring NGC chemical composition and structure. Biocompatibility refers to the ability of NGCs to promote appropriate cellular behaviors, providing molecular and mechanical signaling for nerve regeneration, without eliciting undesirable effects on neural cells and tissues and/or inducing any undesirable local or systemic host response.

Moreover, an ideal NGC should gradually degrade in vivo as the injured nerve regenerates. For this reason, degradable and resorbable materials are generally used to prepare nerve conduits. NGCs should degrade at an appropriate rate which allows the device to withstand the mechanical compression stresses of surrounding tissues till complete nerve regeneration, avoiding guide collapse. In addition, degradation should occur with minimal swelling and foreign body reaction (de Ruiter et al., 2009). Porous guides have been reported to have a decreased degradation rate

compared to not porous guides in the case of bulk degradation phenomena, as porosity decreases autocatalysis effects (Yucel et al., 2010). On the other hand, surface erosion rate is higher in the case of porous guides respect to not porous counterparts since the degradation rate is driven by free surface in contact with aqueous solution (Gopferich, 1996). Mechanical properties of nerve guides should approach the ones of natural nerves to withstand physiological loads: NGCs should bend without kinking and possess a moderate hardness to avoid guide dislocation. Mechanical properties of nerves are reference mechanical properties for nerve guides applications (**Table 1**).

**Table 1. Mechanical properties for natural nerves reported in scientific literature.**

| **Nerve type** | **Elastic modulus (E), MPa** | **Ultimate tensile strength (UTS), MPa** | **Elongation at break (ε), mm·mm$^{-1}$** | **Ref.** |
|---|---|---|---|---|
| Mouse sciatic nerve | 7 | - | - | (Wong et al., 2004) |
| Rabbit tibial nerve | - | 11.7 ±0.7 | 0.385±0.002 | (Nectow et al., 2012) |
| Rat fresh sciatic nerve | 0.580±0.150 | 2.720±0.970 | 0.810±0.114 | (Borschel et al., 2003) |
| Rat acellular sciatic nerve | 0.576±0.160 | 1.400±0.290 | 0.480±0.117 | (Borschel et al., 2003) |
| Intact human nerve | 15.87 ± 2.21 | 6.78±0.57 | 0.61±0.02 | (Dumont and Born, 2005) |
| Extracted Human nerve | 8.19 ± 7.27 | 8.54± 3.37 | 1.64 ±0.34 | (Dumont and Born, 2005) |

Mechanical properties of NGCs depend on the structure of the material, porosity, wall thickness and presence of lumen fillers.

NGC wall permeability should allow the supply of nutrients and oxygen to cells, following device implantation (Gu et al., 2011) and limit the loss of the neurotrophic factors secreted by SCs at the distal stump. NGC wall permeability is affected by the chemical composition of the polymer(s) constituting the guide (hydrophilic/hydrophobic properties; presence of functional moieties able to interact with diffusing molecules), crystallinity degree, wall thickness, porosity degree and pore size. Porosity degree has been reported to be the main factor affecting permeability when using

hydrophobic material-based NGCs, whereas pore size can be tailored to allow vascularisation and to hinder fibroblast infiltration, as well as to limit growth factor outward diffusion (Kokai et al., 2009). The basic structure of a NGC consists of a tubular device with a single lumen, providing the basic functions of a bridging device for nerve regeneration: isolation of the regenerating axons from scar tissue, protection of the regenerating nerve against compression by the surrounding tissue, longitudinal directional guide of regenerating tissue and concentration of the growth factors secreted by SCs in the end stump.

More complex guide designs are available (Daly et al., 2012; Gu et al., 2011) (**Figure 1**), including:

i) Single hollow lumen porous or not porous NGCs providing longitudinally oriented grooves in their lumen surface (Gopferich, 1996) or functionalized with bioactive molecules, such as adhesion proteins (Chiono et al., 2008a), bioactive peptides (e.g. laminin-derived peptides such as YIGSR and IKVAV (Chiono et al., 2009a)) enhancing SCs attachment, proliferation and migration or neurotrophic factors promoting axon growth (Xu et al., 2011). Single hollow lumen NGCs may lead to not complete reinnervation, due to axon dispersion or polyinnervation of different targets by the axons of the same motoneuron. Single hollow lumen NGCs are thus recommended for small lesions (< 30 mm) in the sensory nerves.

ii) Porous or not porous single lumen NGCs, containing fillers as topographical cues enhancing regeneration, mimicking the endoneurial-like structure of autologous nerve grafts. Fillers may include longitudinally aligned fibers (Matsumoto et al., 2000; Wang et al., 2005), porous sponges (Tonda-Turo et al., 2011a) or gels (Ceballos et al., 1999; Nakayama et al., 2007) mimicking the composition and morphology of ECM protein-based intraluminal matrix that naturally supports and enhances the nerve regeneration (Deumens et al., 2010; Gu et al., 2011). Fillers can be functionalized with specific peptides/proteins or neurotrophic factors, as described in recent works (Gu et al., 2011; Jiang et al., 2010; Marquardt and Sakiyama-Elbert, 2013).

iii) Multichannel NGCs mimicking the natural compartment structure of nerves (He et al., 2009; Sun et al., 2012; Sundback et al., 2003). Multichannel NGCs reduce axon dispersion and offer

superior surface area for functionalization and cell adhesion and migration as compared to single lumen NGCs. However, multichannel NGC design reduces permeability and mechanical flexibility. As a result, multichannel NGCs were found not to induce significant functional improvements as compared to single lumen NGCs (de Ruiter et al., 2008).

The aim of this work is that to analyze the recent trends in the design of NGCs, with a focus on the role of the NGC architecture to derive guidelines for optimal NGC fabrication.

*1.5 Evaluation of NGC biological performances*

The evaluation of NGC biological performances has been investigated in a wide range of experimental settings both in vitro and in vivo. In vitro cell tests evaluating vitality, adhesion and proliferation of glial cells (both primary and line cells) are useful to investigate cell response in the first stage of the regeneration process (Tonda-Turo et al., 2011a; Tonda-Turo et al., 2013). Dorsal root ganglia and neurons are mainly applied to measure the induction of axon growth and sprouting (Gnavi et al., 2014). Furthermore, neural stem cells have been used to study the correlation between NGC features and cell differentiation (Gu et al., 2011; Wang et al., 2012) .A variety of animal models (rats, rabbits, cats and dogs), nerve gap lengths and anatomical sites (sciatic or median nerves) have been used to evaluate the performance of NGCs in peripheral nerve regeneration. Furthermore, a large number of assays for assessing the quality of regeneration have been adopted by independent laboratories making difficult to compare the results (Yannas et al., 2007). In general, histological analysis and evaluation of functional recovery are necessary to evaluate the degree of nerve regeneration (Nichols et al., 2005). Functional analysis offers the most direct approach for demonstrating that the regenerated nerve has recovered its motor and sensory function and has correctly innerved the target tissue. Nichols et al. (Nichols et al., 2005) have compiled and tabulated the available motor tests and observational methods applicable to various models of rat nerve injury for studying reinnervation in terms of degree and quality of motor and sensory recovery. In this endeavor, the standardization of experimental conditions and assays could improve

any evaluation of peripheral nerve regeneration. Yannas et al. (Yannas et al., 2007) have reviewed and compared various materials and models for peripheral nerve regeneration concluding that the quality of regeneration can be maximized addressing two critical aspects: (i) the presence of fibroblasts surrounding the NGC and infiltrating within the conduit should be minimized; (ii) the proliferation of SCs and the formation of bands of Bungner serving as tracks for axon elongation should be encouraged.

Experimental models which more closely mimic the environmental and patient clinical conditions will become a fundamental instrument to enlarge the knowledge in the biochemical mechanisms involved in the regeneration process.

## 2. Morphology of nerve guidance channels

A critical aspect in the design of NGC is conferring them the ability to deliver oxygen and nutrients to the regenerating nerve tissues to ensure SCs vitality, especially in the case of large gaps (> 2cm) requiring relatively high recovery times. The first biomaterial based approach to reconnect damaged nerve stumps employed not porous poly(dimethyl siloxane) (PDMS) tubes: these guides were not permeable and their only function was that to avoid the risk of scar tissue ingrowth showing a low axons regeneration and myelinization (Jenq and Coggeshall, 1985). Further studies confirmed the fundamental role of guide permeability to oxygen and nutrient in peripheral nerve regeneration (Jiang et al., 2010; Lu et al., 2009). To maximize the influx of oxygen and nutrient from the interstitial fluid, porous guides have to be used, with proper pore size and porosity degree. Moreover, peripheral nerves are known to be highly vascularized by vasa nervorum along the axon trunk. Nerve trunk (axon bundles) are supplied by blood by arteriae nervorum. Therefore, nerve vasculature is fundamental for nerve regeneration. The need for an adequate wall porosity is contrasted by the opposite need to prevent the infiltration of scar tissue into the NGC lumen and to minimize the outward diffusion of growth factors, secreted by nerve ends. Tube wall pores should be interconnected and with suitable size to allow small molecules permeation; on the other hand

pore size greater than 20 μm causes fibroblast infiltration as reported by Sarazin at al. (Sarazin et al., 2004) and should be avoided. Therefore, optimal pore size for external guide should be comprised in 5-30 μm range and preferable at 10-20 μm range (Dodla and Bellamkonda, 2008; Jiang et al., 2010; Pfister et al., 2007a; Rodriguez et al., 1999; Vleggeert-Lankamp et al., 2007), avoiding growth factors outflow and fibroblast infiltration, although allowing nutrient and catabolyte exchange and endothelial cell migration (vascularization).

In addition, nutrient and oxygen inflow increase with decreasing NGC wall thickness. However, any increase in both NGC porosity (Bian et al., 2009; Yucel et al., 2010) and decrease in the wall thickness reduce the guide mechanical properties, which can compromise NGC ability to face compression stresses.

Therefore, the morphological properties of the guide (wall thickness, porosity degree, pore size) have to be carefully designed to satisfy the NGC requirements in terms of mechanical performance and permeability. To this aim, several conventional and not conventional techniques have been used to obtain porous NGCs with different structural features. Although optimal NGC structural parameters also depend on the guide chemical composition (affecting material mechanical properties), a state-of-the-art analysis of porous NGCs allows the definition of suitable ranges for guide wall thickness, porosity degree and pore size. In the following sections, the main available techniques for the fabrication of porous guides will be reviewed deriving the ranges for optimal NGC structural parameters.

Additionally, in the case of large nerve gap (> 2 cm), a porous inner NGC filler may provide contact guidance for SCs attachment and migration, thus favoring nerve regeneration. The design of inner fillers should consider the needs for a suitable surface composition allowing cell attachment, an adequate porosity for cell migration, the additional possibility to deliver neurotrophic factors to stimulate neuronal outgrowth and a degradation time depending on nerve tissue regeneration rate (which in turn depends on gap size) (Chen et al., 2006; Jin et al., 2013). Biomimetic 3D structures able to reproduce the native structure and size of ECM have been reported to strongly influence the

cell behavior. Interestingly, these biomimetic structures enhance the nerve regeneration process by providing synergistic contact guidance for cell adhesion and topographical cues to direct stem cell fate (Georgiou et al., 2015; Jiang et al., 2012; Lim et al., 2010; Prabhakaran et al., 2009)

In the next paragraphs, a state-of-the art analysis of the most successful luminal NGC fillers will be provided to derive requirements for the design of optimal NGC fillers, in terms of structural, morphological and physicochemical properties.

## 3. Methods for the fabrication of porous conduits and luminal fillers

Conventional and rapid prototyping techniques have been used to prepare porous external conduits and inner fillers for NGCs. Main conventional techniques used in the field of peripheral nerve tissue engineering include electrospinning, porogen-leaching, freeze-drying and solvent- or thermally-induced phase separation. On the other hand, rapid prototyping techniques are a family of emerging software-driven procedures allowing the controlled layer-by-layer fabrication of scaffolds, which have been marginally used in the field of peripheral nerve regeneration up to now.

### *3.1 Conventional techniques*

#### *3.1.1 Electrospinning*

Electrospinning allows the fabrication of randomly or longitudinally aligned nanofibrous substrates with fibre diameters ranging from tens of nanometers to several microns. An electric voltage (± 5÷50 kV) is applied between a collector and a metal capillary needle from which the polymer solution or melt is extruded. The applied electric field induces a surface charge on the polymer solution or melt and the formation of a Taylor cone polymer droplet at the tip of the spinneret. At an electrical potential exceeding a critical value, electrostatic forces overcome surface tension and a polymeric jet stream between the capillary needle and the collector forms. Solvent evaporation (in the case of polymer solutions) or cooling (in the case of polymer melts) occur before nanofibers reach the collector. Electrospun scaffolds have gained increased interest as they have a similar

morphology compared to native ECM (Sill and von Recum, 2008). In addition, electrospinning allows the obtainment of porous polymer substrates with varying porosity degree and mechanical properties, having a high surface to volume ratio, enhancing cell-substrate interactions. Porous guides have been fabricated by electrospinning, having pores with 1-10 μm size, which avoid the infiltration of fibrous tissue into the NGCs, but allow the ingrowth of endothelial cells (vascularisation) when pores have higher size than 5μm (Dodla and Bellamkonda, 2008; Pfister et al., 2007a; Rodriguez et al., 1999). By changing the collector geometry, substrates with different structural and morphological features can be prepared, such as flat membranes (which can be wrapped into tubular guides or cut into stripes used as NGC fillers), mono-layered or bi-layered hollow tubular structures and fiber mats used as inner fillers. **Table 2** collects significant examples of potential use of electrospinning technique in the preparation of NGCs. Exhaustive examples are provided in previous reviews on the use of electrospinning technique for peripheral nerve regeneration (Xie et al., 2010).

**Table 2. Use of electrospinning technique in the field of peripheral nerve regeneration.**

| Application | Final geometry | Collector type | Product characteristics | Ref. |
|---|---|---|---|---|
| Hollow guide | Membrane to be wrapped and longitudinal sutured/glued | Grid/plate | Material: copolymer of caprolactone and ethyl ethylene phosphate | (Chew et al., 2007) |
| | Tube consisting of randomly oriented nanofibres. | Rotating mandrel (with diameter size equal to inner tube size) | Material: blend of poly(L-lactide-*co*-glycolide) and poly-ε-caprolactone (PLGA/PCL)<br>Pore size: from 700 nm to 20 μm | (Panseri et al., 2008) |
| | | | Material: PLGA<br>Average pore size: 1 μm | (Bini et al., 2004) |
| | Tube consisting of nanofibres arranged perpendicularly respect to the tube axis. | Rotating mandrel (with diameter size equal to inner tube size) | Material: PCL | (Teo et al., 2005) |
| | Tube consisting of longitudinally aligned fibres. | Two steel blades (aligned with a gap in between): fibres are deposited aligned along the gap. | Material: PCL | (Teo and Ramakrishna, 2005) |
| | Double layered tubes with the luminal layer having longitudinally aligned nanofibres and the outer layer based on randomly aligned fibres . | Rotating mandrel (with diameter size equal to inner tube size) | Material: poly (L-lactide-co-caprolactone), poly(propylene glycol) and sodium acetate | (Zhu et al., 2011) |
| Random fillers | Cut stripes of membranes with randomly oriented nanofibres | Plate or grid | Material: blend of collagen and glycosaminoglycan<br>Porosity degree: 80.68% | (Timnak et al., 2011) |
| | | | Material: blend of PLGA and PCL<br>Pore size: 9.9±2.0 μm<br>Porosity degree: 64.7±4.4% | (Subramanian et al., 2012) |
| | | Rotating mandrel | Material: chitosan | (Wang et al., 2008) |
| Directional fillers | Mats of longitudinally aligned fibres used as internal fillers. | Rotating mandrel provided with two insulating segments | Material: blend of PLGA and PCL<br>Pore size: 3.7 ± 1.7 μm<br>Porosity degree: 72.0 ± 13.2 % | (Subramanian et al., 2012) |
| | | Two parallel metal rods | Material: blend of polyhydroxybutyrate (PHB) and PLGA | (Yucel et al., 2010) |
| | | Parallel disks (two pole air gap electrospinning system) | Material: PCL<br>Pore size: from 200 nm to 1.8 μm. Porosity degree from 58% to 95%. | (Jha et al., 2011) |

**Figure 2** reports the scanning electron microscopy (SEM) images of electrospun tubes and bundles fabricated according to the procedures reported in **Table 2**.

As evidenced in **Table 2** electrospun substrates have been proposed as both hollow guides and internal filler of NGCs.

For hollow guides preparation, synthetic polymers have been applied and different set up and techniques were investigated. Flat fibrous membranes prepared by electrospinning may be wrapped around the trunked nerve and, then, longitudinally sutured or glued into a tubular guide (Chew et al., 2007). Alternative methods for preparing porous NGCs are based on the electrospinning of polymer solutions onto rotating mandrel collectors with proper diameters according to nerve size (generally 1-2.5 mm) (Bini et al., 2004; Panseri et al., 2008). Finally, advanced procedures have been developed to prepare NGCs with longitudinally aligned fibres by electrospinning (Teo and Ramakrishna, 2005). Teo et al. developed a simple method to collect highly aligned nanofibres using conductive blades placed in line with a gap in between. Nanofibres were collected at the gap with one end at the tip of one steel blade and the other end at the other tip showing highly ordered structure. In the case of polymers with lower mechanical properties, electrospun bi-layered guides have been prepared, with the aim to increase their mechanical performance and structural stability. Zhu et al. have fabricated hollow bi-layered nanofibrous NGCs, with longitudinally aligned nanofibres as the inner layer and randomly oriented nanofibres as the outer layer, based on a blend between poly(L-lactide-co-caprolactone) and poly(propylene glycol) (Zhu et al., 2011). To this purpose, a stainless steel rotating mandrel partially coated with insulating polymer layers was used. Longitudinally aligned nanofibres were first deposited between the two adjacent insulating segments. After complete deposition of the first layer, changes in the electrical field led to the deposition of randomly aligned nanofibres on the previous layer.

The presence of electrospun fibres on NGC wall increases the total surface area available for cell adhesion and introduces contact guidance to cell ingrowth. In vivo tests on rats having a 1cm nerve gap suggested that both randomly oriented and aligned electrospun NGC walls play a significant

role during the initial or early phase of nerve regeneration due to the presence of an increased surface area for cell attachment (Bini et al., 2004; Chew et al., 2007; Panseri et al., 2008). Myelinated axons were found to grow in close proximity of the electrospun fibers during the initial months post-implantation (Chew et al., 2007; Panseri et al., 2008) having smaller area, fibre diameter and density compared to normal nerves (Bini et al., 2004). The effect of morphological and guidance cues were demonstrated on long-term in vivo studies (12 months post-implantation) where aligned nanofibrous nerve conduits showed superior regeneration than randomly oriented nanofibrous conduits and a comparable therapeutic effects to autografts (Zhu et al., 2011).

The possibility to fabricate NGC internal fillers based on nanofibres was investigated by many authors as reported in **Table 2**. Randomly oriented nanofibrous mats were studied as NGC internal fillers to enhance SCs adhesion and proliferation and consequently improve the regeneration process (Subramanian et al., 2012; Timnak et al., 2011; Wang et al., 2008). Additional directional cues can be imparted to SCs by orienting the fibres in the direction of axonal regrowth. Aligned nanofibres were produced by modifying the collector geometry such as using rotating mandrel (Subramanian et al., 2012) or parallel metal rods (Yucel et al., 2010). More complex geometries allowed to obtain three dimensional fibres organization as reported by Jha et al. (Jha et al., 2011).

Cylindrical bundles of aligned nano- to micro-fibres (from 200 nm to 1.8 μm average cross-sectional size) may also be prepared using the "air gap electrospinning" method (Jha et al., 2011). Jha et al. applied this method to prepare PCL fibre bundles. Bundle porosity, calculated by the liquid intrusion method as a function of the feed polymer solution concentration, increased from 58% to 95% with increasing PCL solution concentration from 10% (w/v) % to 25% (w/v), respectively. Complete NGCs was obtained by coating the fibrous bundle through electrospraying of PGA-PLA (50:50) copolymer aiming at reducing inflammatory cell infiltration into the fibre arrays during in vivo implantation.

Compared to empty tubes, NGCs containing both aligned and randomly oriented electrospun fibres showed to promote SC migration into the lesion area creating a growth permissive environment that

enables axons to grow through the lesion site (Timnak et al., 2011; Wang et al., 2008). However, aligned nanofibres mimicking the anisotropic structure of the native tissue facilitate the formation of longitudinal columns of SCs resembling the in vivo architecture of endo-neural peripheral nerve processes (Subramanian et al., 2012). In vivo tests on rats reported that 25% of the axons present in the proximal section reached the distal stumps in a two-fold redaction time if the NGC presents PCL aligned nanofibres as internal filler compared to PCL empty tube (Jha et al., 2011).

Natural polymers such as proteins (gelatin, collagen and silk fibroin) and polysaccharides (chitosan, hyaluronic acid and cellulose) showed higher biomimetic properties than synthetic materials but they are difficult to be electrospun, because of their low solubility in organic solvents, the risk for protein denaturation (Zeugolis et al., 2008) and high viscosity of polysaccharide solutions (Homayoni et al., 2009). Natural polymers have been generally electrospun using highly toxic solvents, such as 2,2,2-trifluoroethanol (Huang et al., 2004), acetic acid (Song et al., 2008), 1,1,1,3,3,3-hexafluoro-2-propanol (Kim et al., 2005) and formic acid (Ki et al., 2005). Electrospun collagen flat membranes have been prepared by Timnak et al. with randomly oriented or longitudinally aligned fibers and have been crosslinked by genipin (Timnak et al., 2011). Collagen solution in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP):Acetic Acid (AAc) (1:1) has been electrospun into longitudinally aligned fibers, using a rotating cylindrical mandrel at an optimal speed of 2600 rpm. Porosity of membranes composed of randomly oriented fibers was around 80%, whereas that of membranes with aligned fibers was not quantified. Membranes with aligned fibers showed closer mechanical properties to those of an intact nerve (**Table 1**) as compared to membranes with randomly oriented fibers (UTS: 5.200±0.600 MPa vs. 0.60±0.10 MPa; ultimate strain: 1.021±0.002 mm/mm vs. 1.14±0.03 mm/mm).

As an alternative to the current electrospinning methods employing organic solvents, the authors of this work have recently fabricated porous glycidoxypropyltrimethoxysilane (GPTMS) crosslinked gelatin membranes, based on randomly oriented electrospun nanofibers with an average diameter of 300 nm, using water as a solvent (Tonda-Turo et al., 2013). Addition of GPTMS crosslinker into

the gelatin solution did not cause any significant increase of solution viscosity during the electrospinning process, as the condensation reaction involved in GPTMS-mediated crosslinking occurred only after complete solvent evaporation (i.e. at the collector). The adhesion and proliferation of glial-like cells on nanofibrous matrices confirmed the potentiality of these scaffolds as substrates for peripheral nerve tissue engineering. Electrospinning of aqueous solution is advantageous as it not only avoids the use of cytotoxic organic solvents, but it also preserves proteins from denaturation. Growth factors may be incorporated into natural polymer nanofibres prepared from water solutions, without losing their bioactivity.

Due to their superior mechanical resistance and peculiar physicochemical properties, such as limited swelling and unaltered mechanical properties in physiological fluids as well as high degradation times, synthetic polymers are ideal candidates for the fabrication of NGCs external conduits. On the contrary, natural polymers have low mechanical resistance, especially in swollen state, and short degradation times but, as an advantage, they display biomimetic characteristics, supporting nerve regeneration. For these reasons, they are generally preferred for the preparation of fillers or inner tube surface coatings for NGCs. Based on these considerations, electrospinning methods collected in **Table 2** could be ideally applied to prepare porous tubular guides based on synthetic polymers, internally coated or filled respectively with a layer or a bundle of longitudinally oriented natural polymer fibres.

The main advantages related to electrospinning technique lies in the possibility to mimic the architecture of natural ECM and fabricate biomimetic structure in an easy manner. Many studies have demonstrated that nanofibres alone support stem cell culture and induce differentiation into neural lineage (Jiang et al., 2012; Lim et al., 2010; Prabhakaran et al., 2009).

*3.1.2 Porogen leaching techniques*

Porous tubular guides or inner fillers can be prepared by blending the selected structural polymers with a second component (porogen), which can be an inorganic compound, such as NaCl or a low-cost polymer removed by dissolution in a selective solvent.

Salt leaching technique has been applied to fabricate NGCs (Kokai et al., 2009). Pore morphology depends on the salt crystals, while porosity is mainly affected by salt weight fraction (Widmer et al., 1998). One drawback of this technique is poor pore interconnection. Attainable pore size varies in the 10-300 μm range (Kokai et al., 2009; Widmer et al., 1998), depending on salt particle size. However, pore size higher than 30 μm allows fibroblast infiltration and neurotrophic factors outflow and is suboptimal for NGCs. Kokai et al. prepared NGCs by dip-coating a mandrel with a NaCl suspension in poly(caprolactone) (PCL) solution, followed by drying and particulate leaching (Kokai et al., 2009). They found out the following optimal structural parameters for NGCs: 80% porosity degree, 10-38 μm pore size and 0.6 mm wall thickness. Due to the relatively high pore size respect to the recommended value (10-20 μm), wall thickness was increased to limit growth factor outward diffusion (studied using lysozyme as model molecule), without affecting glucose permeability (**Figure 3 A-B**).

Blending a structural polymer with a porogen polymer offers the possibility to tailor pore size, by blend composition and processing conditions (Tonda-Turo et al., 2011a; Yucel et al., 2010). Suitable blend compositions for the fabrication of porous guides according to this method are generally below the "co-continuity" region, to afford suitable mechanical properties. After the removal of the leaching polymer component, a not interconnected porosity is thus formed, which does not allow the convective flux of fluids between the guide lumen and the external environment in the first period post-implantation. However, the presence of pores may enhance diffusive exchanges of nutrients and catabolites. In a previous work, Chiono et al. demonstrated that non porous PCL melt-extruded guides were effective for the repair of small (0.5 cm) and medium (1.5 cm) size nerve defects in the peroneal and median nerve of Wistar rats, respectively (Chiono et al.,

2009b). However, PCL guides failed in the regeneration of a 4.5 cm long defect in the median nerve of Wistar rats in a cross-chest experimental model, due to guide detachment from the implantation site, caused by guide rigidity. Further efforts were devoted to increase PCL mechanical flexibility by introducing pores into the tubular conduit wall, which could also increase nutrient diffusion rate favoring nerve regeneration. To this aim, a simple method was developed and recently published by the authors (Tonda-Turo et al., 2011a) based on the preparation of PCL/ poly(ethylene oxide) (PEO) blends by solution mixing, followed by the fabrication of blend tubes by dip-coating/rotating mandrel technique and final PEO selective dissolution leading to porous guides (**Figure 3 C-D**). Different PCL/PEO blend compositions were prepared with PEO content from 10 to 50 wt.%. Porous PCL guides were coded as PCL100i, PCL90i, PCL80i, PCL70i, PCL60i, and PCL50i, respectively for guides prepared from PCL/PEO 100/0, 90/10, 80/20, 70/30, 60/40 and 50/50 wt./wt. after PEO dissolution. Permeability of PCL porous NGCs was determined using FITC-labeled dextran with 4400 Da molecular weight (FD-4, Sigma Aldrich). FD-4 was used as a model molecule to study nutrient diffusion due to its higher Stokes radius (14 Å) compared to glucose (3.8 Å) (Schultz and Solomon, 1961) and NaCl (1.4 Å) (Boyle et al., 1979). Tubes filled with FD-4 solutions were soaked in 10 ml PBS and FD-4 concentration in the incubation medium was assayed by UV-Vis spectroscopy at different time points. The concentration of released FD-4 in the external PBS solution was plotted as percentage respect to the initial concentration filling the tube, as a function of time, for different PCL-based conduits (**Figure 4**). Not-porous PCL NGCs were found to allow the diffusion of small molecules (FD-4): at short time (3-9 hours), the amount of released solute was around 20-25%, it reached 40-50% after 24-48 hours, and it was 70% after 5 days (168 hours). The behavior of PCL90i and PCL 80i was similar to that of control PCL guides, whereas permeability of PCL70i, PCL60i and PCL50i was significantly improved compared to the control after 9 hours. PCL60i was finally selected for the fabrication of guides as it showed partially interconnected pores with 0.90±0.25μm size, suitable mechanical properties (Tonda-Turo et al., 2011a) and optimal permeability to nutrients that guarantee cell survival within the conduits.

The authors tested the same technique on an elastomeric poly(ester urethane) (PU), previously employed to prepare not-porous melt-extruded PU conduits for regeneration of peripheral nerves (Chiono et al., 2011). In detail, PU/PEO (PEO Mw: 100,000 Da; Sigma-Aldrich) 60/40 wt./wt. blend was obtained by solution mixing technique preparing a 5% (wt./vol.) solution using chloroform as solvent. PU/PEO 60/40 tubes were fabricated by dipping a 1.3 mm metal bar into PU/PEO solution; then, the system was air-dried for 30 min. Dipping and drying steps were repeated three times. Prepared tubes were finally dried and, then immersed in distilled water for 48h to dissolve PEO (PU60i). PU60i tubes were analyzed for their morphology evidencing a not-porous structure (**Figure 3 E-F**). Due to PU elastomeric properties, macromolecular chains rearranged their conformation after PEO removal resulting in a compact structure instead of a porous one (as observed for PCL). As a conclusion, PEO leaching technique can be successfully applied to glassy or semi-crystalline polymers due to the limited mobility of macromolecular chains, whereas it cannot be applied elastomeric polymers.

*3.1.3 Freeze drying*

Freeze-drying is an advantageous technique to prepare porous substrates, as it does not require the use of porogens. The procedure is simple: initially, water based polymer solutions are prepared, then they are frozen and, finally, they are freeze-dried for ice sublimation. Natural polymers have been widely used due to their water solubility and different pore micro-architectures have been obtained (Stokols and Tuszynski, 2004). Freeze-dried substrates based on natural polymers have been generally used as porous NGC luminal fillers, showing interconnected pores for cell infiltration and tissue growth. For instance, an luminal NGC filler based on freeze-dried genipin-crosslinked gelatin (GL/GP) has been developed by the authors (Tonda-Turo et al., 2011a). The GL/GP porous matrix showed an interconnected porosity with pores of around 62 μm size, which were suitable for glial-like cells adhesion and proliferation (**Figure 5A**).

By modified freeze-drying approaches, porous matrices with oriented pores have been obtained. Collagen/heparin sulfate (100/1 wt./wt.) sponge-like fillers with oriented pores were prepared by freeze-drying a collagen/heparin sulfate solution inserted into a tube with 3 mm inner diameter, previously immersed in liquid nitrogen at a rate of $2 \cdot 10^{-5}$ $m \cdot s^{-1}$ (Wang et al., 2012). Scaffold pores size was comprised between 78 and 109 μm whereas porosity degree was about 89%. Sponges were found to support the adhesion and proliferation of neural stem cells, which distributed along the pore direction. The use of heparin sulfate was proposed for the incorporation of heparin-binding growth factors, such as FGF, stimulating axon regeneration (**Figure 5B**).

Bozkurt et al. prepared collagen-based nerve guides (Perimaix) having longitudinally oriented channels with an average diameter of around 50μm, using a patented unidirectional freezing process (Bozkurt et al., 2012). The channels were continuous from one end to the other one of the NGC and stabilized by collagen strands perpendicular to the longitudinal axis. Schwann cells were cultured within the Perimaix scaffold, which was then implanted for the treatment of a 2 cm long defect in the rat sciatic nerve. The longitudinally oriented channels supported directional glial cell migration and histological micrographs confirmed that SCs were aligned in a columnar fashion within the orientated micro-channels at one week and 6 weeks after implantation. The regenerative properties of SC-cultured Perimaix NGCs were similar to those of autografts, after 1 and 6 weeks implantation time. However, future Perimaix strategies should consider the need to develop a tubular component replacing damaged epineurium (**Figure 5 C-D**).

Ao et al. applied an unidirectional temperature gradient to induce uniaxial phase separation to fabricate aligned channels as internal filler of NGCs (Ao et al., 2006). Multimicrotubule chitosan conduits (M-conduits) were fabricated starting from a chitosan hollow guide that was inserted into a properly-dimensional styrofoam pedestal, and filled with chitosan acetic acid solution. Then, the styrofoam pedestal was rapidly covered with a pre-cooled stainless steel cover plate, and placed in a freezer (-20 to -80°C). Thanks to the presence of the styrofoam pedestal the phase separation process occurred from the top end to the bottom end of the chitosan solution. The frozen samples

were freeze-dried to remove iced solvent and then immersed for 10–20 min into 2% (w/v) sodium hydroxide (NaOH) solution to neutralize the remaining acetic acid. Oriented microtubules were formed and their diameter was controlled by adjusting the polymer concentration and the cooling temperature. In details, microchannel diameter can be reduced by increasing solution concentration and decreasing freezing temperature. Using a chitosan acetic acid solution with 4% (w/v) concentration and a freezing temperature of -40°C, microchannels with hexagonal section were obtained. Their size ranged from 10 to 150 μm and about 73% of the microchannels showed a diameter of 30–90 μm. In vitro cell tests were performed using mouse neuroblastoma cell line (Neuro-2a cells) and confirmed that this multitubular NGC is promising as scaffold for peripheral nerve regeneration thanks to the presence of a proper porosity for fluid exchange and topographical cues for SC 3D organization.

Although mechanical properties of natural polymers are generally poor to allow their use as materials for external guides, crosslinking may not only reduce water absorption and degradation rate but even increase mechanical performance (Tonda-Turo et al., 2011b). In this context, the authors fabricated porous chitosan (CS, medium molecular weight, 75%-85% deacetylated, Sigma Aldrich) membranes, by freeze-drying CS solutions in 0.5 M acetic acid with 2.5% wt./vol concentration. CS substrates display low mechanical strength and undergo significant swelling in wet state (physiological conditions) which both limit their use as NGC in clinical applications. Aiming at improving CS membrane mechanical properties and their shape stability in physiological conditions, the authors studied the effect of a silane agent (GPTMS) used as CS crosslinker. GPTMS was added to CS solution at 75% wt./wt. concentration with respect to CS (the amount of GPTMS was optimized to have the maximum crosslinking degree of CS chains). The resulting solution (CS/GPTMS solution) was kept under stirring at 50°C for 1 hours. The porous membranes were fabricated by pouring the CS/GPTMS solution (3ml) into 60 mm Petri dishes followed by freezing at -20°C for 24 hours. The frozen samples were subsequently freeze-dryed at -20°C for 24 hours. The acetic acid was then neutralized by rinsing in a 0.25 M NaOH solution followed by ten

washing steps in distilled water. A second freeze-drying process was carried out to maintain the membrane porous structure. The membranes were highly porous (**Figure 6**) with interconnected pores having a mean pore size of 13.4±9.6 μm. Compressive stress–strain curves for the CS and CS/GPTMS freeze-dried substrates were measured using cylindrical specimens with 1 cm diameter and 1 cm height in a MTS QTest/10 device. All the specimens were compressed at an uniform strain rate of 10 mm/min and the compressive force was applied along the height of the samples. Three specimens for each kind of material were tested. Young's modulus (E) and collapse modulus (E*) were measured from the stress-strain curves (Kanungo et al., 2008). Crosslinked samples showed increased values of E and E* respect to uncrosslinked ones: E and E* were 559.0±51.2 kPa and 100.6±13.2 kPa for CS and 1012.8±87.8 kPa and 116.3±8.2 kPa for CS/GPTMS. The statistical significant increase of E and E* values for CS/GPTMS samples (*$p<0.05$) was a consequence of the mechanical reinforcement associated with crosslinking process. Furthermore, qualitative tests indicated the superior elastic behavior of CS/GPTMS membranes which facilitates wrapping of the porous membrane without plastic deformation. The crosslinked CS/GPTMS porous membranes are expected to be easily wrapped around the trunked nerve stumps, allowing their use for the preparation of NGCs during the surgical intervention, with the more appropriate size depending on the treated nerve diameter.

*3.1.4 Non-solvent induced phase separation (NIPS) and thermally induced phase separation (TIPS)*

Phase separation-based methods convert a homogeneous polymeric solution into a biphasic system in which the polymer-rich phase constitutes the scaffold matrix, whereas the polymer-poor phase forms the matrix pores. The phenomenon is driven by inducing a thermodynamic instability through a change in composition or temperature. Phase separation techniques applied in TE include non-solvent induced phase separation (NIPS) and thermally induced phase separation (TIPS). In NIPS, phase separation is caused by the contact of the polymer solution with a non-solvent, leading to polymer precipitation. In TIPS, a polymer solution is prepared at higher temperature than upper

critical solution temperature; then, it is quenched to induce phase separation. The solvent is eliminated through immersion of the system in a polymer non-solvent.

Phase separation techniques has been explored in order to produce NGCs with interconnected porous structure through one simple process that is scalable, fast and controllable.

Oh et al. applied NIPS to prepare asymmetrically porous poly(lactic acid-co-glycolic acid) (PLGA) NGCs (Oh et al., 2008). An alginate hydrogel rod was immersed into a PLGA solution in glycofurol and Pluronic F127 was used as hydrophilic additive. Phase inversion was carried out by immersion in a water bath. Prepared NGCs (diameter of 1.5 mm and wall thickness of 0.4 mm) showed asymmetric column shaped porous structure, with nanosized pores on the inner tube surface (~50 nm) and microsized pores on the outer tube surface (~50 μm) (**Figure 7A)**. The developed NGCs were tested in vivo removing 10 mm of sciatic nerve. Immunohistochemical analysis was performed at 1, 2, 4, and 8 weeks post-implantation while histological evaluation at was carried out at 12 and 24 weeks post-implantation. Compared to non porous silicone tubes, asymmetrically porous PLGA tubes showed a faster axonal growth (axons reached the distal stump after about 4 weeks) and larger axon diameter and thicker myelin sheath at 12 and 24 weeks.

Hsu et al. combined microprinting and NIPS to obtain poly(D,L-lactic acid) (PLA) substrates to be rolled into NGCs, exhibiting microgrooves on their inner surface for directional guidance and asymmetric porosity for providing asymmetric permeability (Hsu and Ni, 2009). PLA solution in dioxane was cast on a patterned PDMS mold. Then, the samples were immersed in ethyl alcohol/water solutions with different concentrations (95, 40, 20 vol. %). The average pore size increased while the groove integrity decreased with decreasing ethyl alcohol concentration. Asymmetric pores formed when 40 and 20 vol. % ethyl alcohol was used as non-solvent: on the microgrooved surface, pores showed 5 μm size, while on the unpatterned surface, elliptical pores were present, with 10 μm x 5 μm and 20 μm x 10 μm size, for samples obtained using 40 vol.% and 20 vol.% ethyl alcohol, respectively. Substrates obtained using 40 vol.% ethyl alcohol showed directional selectivity in bovine serum albumin (BSA) permeability. Furthermore, in vivo results

obtained using the rat sciatic nerve model with 10mm nerve defect showed a higher degree of myelination at 4 weeks and at 6 weeks in the asymmetric conduits with surface microgrooves than on asymmetric conduits without surface microgrooves highlighting the importance of wall porosity on the regeneration process.

The authors combined dry-jet-wet spinning technique and NIPS to prepare porous poly(3-hydroxybutyrate-co-3-hydroxyvalerate)/poly(ε-caprolactone) (PHBHV/PCL) hollow conduits for biomedical applications (Chiono et al., 2008b). For PHBHV/PCL blends with PCL content ≤ 60 wt.%, continuous hollow fibers were obtained with low porosity degree and rough porous inner and outer surfaces (**Figure 7C**). Pore size was lower than 10 μm. Fibers with the same composition and tailored size (obtained by properly varying the spinneret size and non solvent flow rate into the inner cavity) could be potentially exploited for peripheral nerve regeneration.

Sun et al. developed a multi-channeled nanofibrous poly(L-lactic acid) (PLLA) NGC through injection molding and TIPS techniques (Sun et al., 2012) to fabricate a multi-structural guide having porous external wall and internal microchannels (**Figure 8**). PLLA solutions in tetrahydrofuran with 6-12 % wt.·vol.$^{-1}$ concentration were prepared and injected into molds, followed by phase separation at -80°C for 12 h. Subsequently, the solvent was exchanged with ice-cold distilled water. Resulting scaffolds were negative replica of the mold characterized by internal microchannels and channels wall consisted of interconnected nanofibers with 157-161 nm size. Fiber length and scaffold porosity decreased with increasing PLLA solution concentration from 1250 nm to 556 nm and from 92 to 85%, respectively. The porous nanofibrous channeled matrix was proposed as luminal filler to guide the regeneration of oriented axon bundles. Preliminary in vitro tests using rat adrenal pheochromocytoma cell line (PC12 cells) showed an increased cell adhesion on conduits with inner fillers.

*3.1.5 Injectable hydrogels as fillers*

In peripheral nerve tissue engineering, hydrogels are applied as NGC fillers, serving to physically bolster the nerve conduit lumen, and to act as delivery vehicles for bioactive molecules and cells (Lin and Marra, 2012). Tubes filled with fibrin matrices (Williams et al., 1987), laminin containing gels (Madison et al., 1985), collagen (Ciardelli and Chiono, 2006; Cordeiro et al., 1989), hyaluronic acid (Seckel et al., 1995) have shown faster axonal regeneration compared to empty tubes or tubes containing physiological saline solution. Despite many studies demonstrated that NGC fillers can enhance the regeneration process, hydrogels with unsuitable physicochemical properties could represent a physical barrier to axonal growth impeding regeneration. Therefore hydrogels to be applied as internal fillers for NGCs should be designed to have suitable morphological properties (i.e. to have a proper intrinsic porosity to allow nutrient diffusion) and a degradation rate matching the nerve regeneration rate to avoid any risk of hindering axon re-growth.

Many hydrogel types with different chemical and physical properties have been developed over the last several decades from a wide variety of materials. In the past, many applications in peripheral nerve regeneration have made use of synthetic water soluble polymers such as poly(acrylic acids), poly(acrylamides), poly(ethelene oxide), poly(vinyl alcohols), and poly(vinyl pyrrolidones) (Lyons et al., 2009; Mahoney and Anseth, 2006; Martens et al., 2003). Synthetic water soluble polymers are advantageous due to their mechanical properties, processability and chemical reproducibility but they lack ECM biomimetic properties which are crucial for cell adhesion and proliferation.

To improve hydrogel biological response, natural polymers such as hyaluronic acid, fibrin and chitosan, have been widely used in tissue engineering applications (Balgude et al., 2001; Crompton et al., 2007; Meena et al., 2007; Sakiyama et al., 1999) as they provide biomimetic chemical and morphological cues.

The use of water soluble hydrogels as NGC fillers allowed the loading of biomolecules and cells within the hollow guide. Localized growth factor release and cell-based therapy have been recently

identified as advanced approaches that can improve the NGC performances and in vivo outcomes to overlap the gap between biological response of artificial graft compared to autograft.

Authors have recently developed a natural origin injectable hydrogel as filler for NGCs (Tonda-Turo et al., 2014). The developed agar/gelatin (A/GL) hydrogels crosslinked using genipin (A/GL_GP) showed shear-thinning behaviour allowing their injection through a syringe needle into a hollow guide during surgery (**Figure 9**). *In vitro* cellular tests using mouse embryonic fibroblast cell line (NIH3T3), neonatal olfactory bulb ensheating cells (NOBECs) and a Schwann cell line (RT4-D6P2T) confirmed cell adhesion, proliferation and viability. Furthermore, three-dimensional migration assay confirmed the capability of the developed hydrogel to allow cell migration by a porous structure that reduces the risk of axonal growth obstruction.

The developed hydrogel was studied as a drug delivery system for growth factor localized release. The mild conditions employed for the preparation of A/GL_GP hydrogel (low temperature and physiological pH) could ideally allow the incorporation of growth factors, as chemotactical cues enhancing the regeneration process (Pfister et al., 2007b). Vascular endothelial growth factor (VEGF) was added to the hydrogel prior to gelation and VEGF release kinetics and bioactivity after release were analyzed through ELISA kit and in vitro tests using human umbilical vein endothelial cells (HUVECs), respectively. Results from in vitro tests showed that GL-based hydrogel system was suitable for incorporation and release of bioactive VEGF, which could be exploited to induce capillary-like tube formation and axonal outgrowth (Gnavi et al, 2014). Furthermore, drug release kinetics could be modulated in multifunctional systems where bioactive agents were loaded in hollow glass fibers made of resorbable resorbable phosphate glass ($50P_2O_5$–$30CaO$–$9Na_2O$–$3SiO_2$–$3MgO$–$2.5K_2O$–$2.5TiO_2$ mol%) dispersed in a A/GL_GP hydrogel (Novajra et al., 2014). Hollow fibers can be used both as a directional fillers and carriers with high surface area for drug delivery.

In addition, thanks to their high water content and their structural similarity to many soft tissues, hydrogels have been used to encapsulate cells in a three dimensional environment (Suri and Schmidt, 2010). In this context, hydrogels act as a vehicle for cell-based replacement therapy

reducing drawbacks related with transplantation of matrix-free cells (e.g. dislocation, low survival and poor 3D organisation).

*3.1.6 Cell-based therapies for axon regrowth*

Recently, cell-based therapies have been identified as promising strategies for treatment of injured peripheral nerve. The presence of transplanted cells is important for the formation of oriented path for axonal growth and orientation (bands of Büngner) (Galla et al., 2004; Suri and Schmidt, 2010). Galla et al. (Galla et al., 2004) studied the effect of fibrin hydrogels as vehicle for transplanted cells. Schwann cells were inserted into PCL tubes in three different ways: as a non-structured cell suspension or embedded in a three dimensional matrix containing or not the neurotrophic factor LIF (leukemia inhibitory factor). In vivo response of the three different systems was evaluated in the repair of a 10 mm gap in the buccal branch of the rat facial nerve. Histological and morphometric analyses of the implants were performed after four weeks implantation, revealing that three dimensional fibrin/Schwann cells matrix enhanced the quantity and the quality of peripheral nerve regeneration through PCL conduits. Furthermore, the presence of LIF prevented hyperneurotization. Hydrogels composed by ECM components, including proteins and glycosaminoglycans, have been developed as cell-housing devices, in an attempt to recapitulate ECM's structural, biological, and mechanical properties and to fabricate a biomimetic environment for cell incapsulalion. In the native environment, SCs are surrounded by endoneurium, the ECM of neural tissue, which is composed mainly by hyaluronic acid (HA), collagen IV, and laminin. Therefore, hydrogels for SCs encapsulation should mimic the neural ECM composition in order to facilitate the cell/scaffold interactions and to enhance cell survival, viability and maintenance of typical glial cell spindle and star-shape morphology. Suri et al.(Suri and Schmidt, 2010) have designed and tested a collagen and HA interpenetrating polymer network (IPN) as 3D ECM-mimicking scaffold for SCs encapsulation. Moreover, to further analyze the influence of hydrogel composition on cellular behaviour, laminin was added to collagen/HA hydrogels. Laminin was selected since it has been reported to enhance

the neurite extension and influence SC migration (Thompson and Buettner, 2001). Collagen/HA hydrogels supported SC viability for 2 weeks and encapsulated cells were able to proliferate and spread within the gel especially when a high cell density ($8x10^6$cells/ml compared to $2x10^6$cells/ml) was loaded into the hydrogel favouring cell-cell interactions. Morphological observations through SEM reveal that cells were able to remodel the hydrogel by secreting ECM molecules. Finally, the amount of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) released from encapsulated SC was quantified to evaluate cellular functional activity. Laminin-containing hydrogels induced significantly higher NGF and BDNF secretion demonstrating the importance of hydrogel composition to loaded SC survival and bioactivity. Finally, SEM micrographs of laminin-containing hydrogels showed the alignment of loaded SCs in a preferential direction forming tubular structures similar to the bands of Büngner. These findings suggested that hydrogel composition mimicking native ECM are advantageous in terms of cell organization and functionality.

Concerning cellular therapy, the use of hydrogel as a cell-laden construct is advantageous since they protect encapsulated cells from the immune system in the in vivo environment and ensure the homogeneous distribution of cells in the injured zone. On the other hand, to achieve an effective and prolonged cellular therapy, hydrogels should be designed to protect transplanted cells against death-mediating mechanisms (such as inflammation and oxidative stress) and to be permeable to nutrients to guarantee long term cells survival (Ritfeld et al., 2014). Permeability is an essential parameters in cell-laden hydrogel design to enable oxygen and nutrients transport and depends strongly on polymeric material chemistry as well as on network structure of the hydrogels (Nafea et al., 2014). Briefly, hydrogel mesh size is correlated with hydrogel permeability to solutes of different sizes and shapes since it provides an average measure of the space available between the macromolecular chains. This intrinsic nano-porosity can be associated to macroporosity to facilitate nutrient transfer, vascularization and cell survival (Desai et al., 2012). Traditional techniques developed for introducing macroporosity into hydrogel-based scaffolds generally result in spherical

macropores formation and fabrication conditions that are potentially cytotoxic (use of organic solvents, high temperature, non-physiological pH). To overcome these limitations, Hammer et al. (Hammer et al., 2014) assessed a new and cell-friendly method to fabricate 50–200 µm size microchannels within the 3D structure of gelatin-based hydrogels. Calcium alginate (Ca-Alg) microfibers cointaining cells were fabricated by wet-spinning and then encapsulated within photocrosslinkable hydrogels composed of methacrylated gelatin (Gel-MA). The ability of alginate to dissolve upon exposure to ethylenediaminetetraacetic acid (EDTA) was exploited to form the microchannels inside the Gel-MA hydrogel. After EDTA treatment, cells within Ca-Alg hydrogels were released and adhered to the microchannel walls forming mono-layered cell colonies along the lumen wall of microchannels. The obtained three-dimensional microchannel-like porosity are recommended for many tissue engineering applications especially in the peripheral nervous system where nerves bundles are characterized by microfibrous structures. Furthermore, the possibility to homogenously distribute cells inside the microchannels prior to in vivo implantation is an important feature for the development of cellularized scaffolds able to strongly enhance the regeneration process.

Recently, mesenchymal stem cells (MSCs) have been proposed as transplantable cells in alternative to glial cells. MSCs have also been reported to differentiate in vitro and in vivo into non-mesodermal cell types such as neurons and astrocytes (Phinney and Isakova, 2005; Scuteri et al., 2011). The presence of MSC into porous PCL tubes have been reported to increase the number of myelinated fibers compared empty tubes in the treatment of median nerves (Oliveira et al., 2014).

### *3.2 Rapid prototyping techniques*

The fabrication of novel microstructured scaffolds involves innovative techniques based on rapid prototyping (RP) that is an emerging manufacturing process used to produce complicated 3D structures automatically using computer-aided designs (CADs). Several RP techniques are now available, such as membrane lamination, ink-jet printing and fused deposition modeling, and can be

applied to process biocompatible and biodegradable polymers. RP techniques allow the preparation of scaffolds with complex controlled geometry, having a porosity degree up to 90%, interconnected pores and reproducible pore size and density distribution (Leong et al., 2003). RP processes have been recently adapted and utilized to form nerve conduits with precise dimensions and complex internal structures (Cui et al., 2009; Radulescu et al., 2007; Yamada et al., 2008). An ink-jet microdispersing system was developed by Radulescu et al. (Radulescu et al., 2007) for preparing a cylindrical shape scaffold that can be applied as NGCs. A PLA/PCL 80/20 copolymer was selected since it displayed good cell adhesion and proliferation of human embryotic kidney cells genetically modified to produce nerve growth factor (hNGF-EcR-293) acting as nerve growth factor release system when implanted in vivo. The presence of nerve growth factors at the injured site can strongly enhance the regeneration process (Chung et al., 2011). After selecting the appropriate polymer for cell survival, authors set up the solution parameters (concentration and solvent) to obtain an injectable solution and adjusted the ink-jet microdisperser parameters (motion speed, layer dimension, printing pattern) to fabricate the required scaffold shape. Finally, cylindrical shape conduits based on PLA/PCL 80/20 were fabricated showing the ability to maintain hNGF-EcR-293 vitality.

FDM technique was optimized by Yamata et al. (Yamada et al., 2008) to be applied for tissue engineering scaffold fabrication. A novel method for three-dimensional microstructures fabrication was developed based on FDM technologies. Biodegradable aliphatic polyesters (PLA, PGA and PLGA) were processed avoiding the use toxic solvents and microstructures of different shapes were obtained having a resolution of 45μm. PC12 cells showed similar morphology and proliferation rate when cultured on microfabricated PLA substrate compared to standard plate.

Cui et al. (Cui et al., 2009) prepared a double layer polyurethane-collagen NGC via a double nozzle, low temperature, deposition manufacturing (DLDM) system, followed by post-processing (by thermally induced phase separation and freeze-drying). One nozzle was fed with collagen solution to obtain the inner guide layer, while the other nozzle was fed with a polyurethane solution in

dioxane to form the external NGC layer. Porosity degree of the external layer was increased from 74% to 82% with increasing polyurethane solution concentration from 8 to 12% (w/v). The inner collagen layer showed a fibrous structure with nanosized filaments, while the outer polyurethane layer showed pores with size from 15 to 25 μm. DLDM technique was advantageous as it allowed the obtainment of a double layered NGC with a regular and reproducible architecture in a single process. Moreover, low temperature processing is advantageous as it could allow the incorporation of growth factors and cells during scaffold fabrication.

In-depth biological characterization of NGCs obtained using rapid prototyping techniques is still lacking and further evaluations are required to assess their applicability in peripheral nerve regeneration.

## 4. Design trends

Current available and FDA approved bioresorbable NGCs are not recommended for larger gaps than 3 cm (Kehoe et al., 2012) and their biological performances are inferior to autograft that remains the gold standard treatment for bridging approaches. The effective permeation of nutrients and prevention of fibrous scar tissue invasion as well as the good mechanical strength of the tube to maintain a stable support structure for the nerve regeneration are the fundamental features of artificial guides to be successfully applied as NGCs.

The current trend of peripheral nerve tissue engineering is to design advanced NGCs having internal structures providing topographic, chemotactic and haptotactic cues able to increase the axon regenerating rate, thus avoiding or minimizing end-organ (e.g. muscle) atrophy. In the case of large nerve gap (> 2cm), NGCs design should be inspired by biomimetic principles: NGCs should resemble the native architecture of the peripheral nerve and provide directional guidance to regenerating axons. Furthermore to improve the biological outcomes, new strategies to enhance the regeneration process based on cell-loaded fillers are under investigation.

Summarizing the features described above, the external guide of NGCs should satisfy the following requirements:

- Mechanical properties close to those of a human nerve: E values in 8-16 MPa range, UTS values comprised between 6.5-8.5 MPa and ε of around 0.6-1.6 $mm \cdot mm^{-1}$ The guide should be resilient in order to be sutured at the nerve ends without any decrease in its mechanical performance. In addition, the guide should be resistant to the compression stresses deriving from the formation of external fibrotic tissue; finally, it should be able to bend without kinking.
- Porosity degree higher than 50% with an optimal value of around 70%, allowing the guide to be permeable to nutrients and waste products. Porosities higher than 80% generally cause guide mechanical instability (Kokai et al., 2009; Wen and Tresco, 2006).
- Pore size of around 5-30 μm (with optimal values of 10-20 μm), to allow nutrient and waste products permeation and the infiltration of blood vessels and to protect the regeneration environment from the infiltration of scar tissue, although avoiding the outflow of the growth factors secreted by the distal nerve stump.
- Guide wall thickness selected on the basis of the mechanical requirements, target degradation rate, permeability specifications and easy suturability. Guide thickness up to 200 μm allows nutrient diffusion, while a value of 600 μm is the maximum guide thickness value as it is associated with minimal diffusion (Kokai et al., 2009). Guides have been generally prepared with a thickness of around 100-300 μm.
- Diameter of the conduit tailored to the nerve size, avoiding nerve compression caused by degradation-induced swelling. A lower NGC diameter than nerve size may cause chronic nerve compression resulting in nerve damage (Mackinnon et al., 1984). On the other hand, a larger NGC diameter than nerve size may fail to support the regenerating nerve.
- Suitable NGC length, to facilitate the bridging of the nerve gap, without tension.
- Tailored degradation rate respect to the nerve tissue regeneration rate.

- Limited swelling degree during degradation to avoid reduction in the conduct lumen.

The NGC internal filler should satisfy the following requirements:

- It should provide *haptotactic cues* for SCs adhesion and migration. For this reason, the use of natural polymers naturally constituting ECM (e.g. collagen) or with similar composition to ECM components (e.g. gelatin, chitosan), as well as bioactive peptides (e.g. IKVAV, YIGSR), is recommended for filler materials, as they can specifically interact with SCs via integrin receptors.
- It should be porous with porosity degree of around 60-80% and pore size of 30-50 μm to allow SCs migration from the proximal to the distal end.
- It should be degradable with a rate depending on axon regeneration rate not to obstacle axon growth.
- Swelling degree should be limited to avoid compression of regenerating axons.
- It should additionally provide directional guidance (*topographical cues*) to further enhance regeneration rate. To this purpose, the introduction of fillers based on aligned fibers or provided with longitudinally oriented channels could be advantageous.

NGC presenting the above listed features have been shown to achieve nerve regeneration at the injured site with regenerated nerves characterized by cables having a smaller area, fibre diameter and density compared to normal nerves. The encouraging results obtained in the last decade with NGCs still show inferior performances than autograft. To overcome these limitations, advanced NGCs than can revolutionize the biological performances of artificial grafts should introduce growth factors or cells into the conduit lumen. Innovative NGCs should be designed:

- to provide *chemotactic cues* by the incorporation and release of growth factors.
- To introduce cells into the guide lumen, mainly exploiting the paracrine effect of the implanted cells towards nerve regeneration. Typically, autologous SCs or mesenchymal and neural stem cells have been tested (Gu et al., 2011). Implanted cells keep viable for some weeks, depositing their ECM and secreting growth factors which can stimulate regeneration.

However, their integration or differentiation into glial cells have never been demonstrated. In addition, the use of cellularised fillers (Galla et al., 2004; Suri and Schmidt, 2010; Thompson and Buettner, 2001) make method industrialization and translation into clinics difficult, with high cost of the final product.

Previous reviews have been focused on materials used for NGCs, including the functionalisation with peptides, growth factors or the use of cells within the tube lumen (Gu et al., 2011). The aim of this work was that to provide an overview on architectural design of NGCs, which is currently lacking in the scientific literature. The manufacturing techniques for the preparation of NGCs are listed in **Table 3** together with commonly attainable architectural features (porosity degree and pore size).

**Table 3. General features of the manufacturing techniques employed to fabricate NGCs: main process parameters and average porosity degree and pore size of the final NGC substrate**.

| **Technique** | **Main process parameters** | **Porosity degree (%)** | **Pore size (μm)** | **Ref** |
|---|---|---|---|---|
| Electrospinning | • Solution concentration<br>• Electric field<br>• Collector type<br>• Syringe tip-collector distance<br>• Flow rate | 58-95 | 1-10 μm | (Chew et al., 2007; Wang et al., 2008) |
| Porogen leaching | • Porogen (salt or polymer) amount<br>• Size of porogen salt crystals<br>• Compatibility of polymer blends | 80-90 | • 0.9-5 μm (poly(ethylene glycole), PEG)<br>• 10-300 μm (NaCl) | (Kokai et al., 2009; Matsumoto et al., 2000; Tonda-Turo et al., 2011a; Widmer et al., 1998; Yucel et al., 2010) |
| Freeze-Drying | • Solution concentration<br>• Freezing temperature<br>• Temperature gradient during freezing | ~ 90 | 10-300 μm | (Bozkurt et al., 2012; Tonda-Turo et al., 2011a; Wang et al., 2012) |
| Thermally induced phase separation (TIPS) | • Solution concentration<br>• Temperature of phase separation<br>• Temperature gradient during cooling | 85-90 | 15-140 μm | (Ao et al., 2006; Sun et al., 2012) |

| | | | | |
|---|---|---|---|---|
| Non solvent induced phase separation (NIPS) | • Solution concentration<br>• Solvent/Non-solvent pair | - | 50 nm -50 μm | (Chiono et al., 2008b; Hsu and Ni, 2009; Oh et al., 2008) |
| Rapid Prototyping (RP) | • Unit cell geometry and size<br>• Layer assembly method<br>• Layer number and their reciprocal orientation | Controlled and reproducible: 75-90, generally | As required | (Cui et al., 2009; Radulescu et al., 2007; Yamada et al., 2008) |

On the basis of **Table 3** data and previous discussion, electrospinning, porogen leaching and NIPS are suitable manufacturing techniques for the fabrication of external guides having suitable mechanical properties and permeability, whereas inner fillers may be prepared by freeze-drying, electrospinning or TIPS where aligned and oriented porous structures showed improved results compared to randomly oriented pores due to their biomimetic morphology. In addition, injectable hydrogels are also advantageous as NGC fillers for both growth factors release and cell encapsulation (cellular therapy).

Complex NGCs consisting of an external guide and a filler may be assembled by different procedures. For instance, aligned electrospun fibers may be placed on the surface of porous membranes, which are then rolled and longitudinally glued (Okamoto et al., 2010; Yucel et al., 2010). This technique allows the preparation of composite conduit during surgery, adapting NGC diameter to that of the regenerating nerve. Alternatively porous guides may be prepared and then filled with freeze-dried porous matrices or injectable hydrogels (Gu et al., 2011).

Composite conduits entirely based on natural polymers, such as collagen, have been shown to have higher degradation rate than that required for the regeneration of severe (> 30 mm) nerve defects (Okamoto et al., 2010). Ideally, the external guide should be based on a slowly degrading and mechanically resistant material, such as a biocompatible synthetic polymer. On the other hand, the inner matrix should be based on natural polymers, providing haptotactic cues for the adhesion and migration of SCs.

Finally, RP methods are emerging techniques for NGC manufacturing, allowing computer-driven design of scaffolds with desired architecture. RP allows the obtainment of structures with reproducible geometry, for easy industrialization. However, the relatively high cost of this manufacturing technique currently limits its diffusion.

A wide range of in vitro and in vivo experimental settings to test developed NGCs have been reported making difficult a direct comparison of the different results. Therefore, the adoption of standardized experimental conditions and assays should be encouraged for a correct interpretation of the biological response to the implantation of different NGCs.

## 5. Conclusions

The combination of different cues in a single device is expected to maximize the influence of the guidance signaling and could be promising for the repair of large nerve gaps. The external guide should satisfy different requirements: it should display biomimetic mechanical properties, 50-80% porosity degree with ideal pore size of 5-30 μm and tailored degradation rate. Biocompatible synthetic polymers are the ideal candidate materials to be used for the preparation of external tubular conduits for NGCs, due to their high mechanical properties and shape stability in physiological media, while NIPS, particulate leaching and electrospinning are the preferred methods to satisfy architectural requirements of tubular guides. For instance, the authors proposed the preparation of PCL guides by PEO leaching technique, having pores with 0.9 μm size, biomimetic mechanical properties and suitable permeability (Tonda-Turo et al., 2011a). Alternatively, the authors proposed the use of highly crosslinked natural polymers as external tubular conduits which can be easily rolled into tubes with desired size.

Inner filler for NGCs should satisfy the following main requirements: porosity degree of 60-80 %, pore size of around 30-50 μm, suitable degradation rate respect to axon regeneration rate and limited swelling to avoid axon compression. Natural polymer inner fillers for NGCs can be prepared in the form of fibers, gels and sponges; providing haptotactic and topographical cues to

regenerating axons. Aligned electrospun mats may be easily inserted into guides prepared from rolled membranes; on the other hand, gels may be injected into tubular conduits, while sponges may be prepared by freeze-drying or TIPS within the tubular conduits. The authors proposed different types of gelatin based fillers: electrospun gelatin nanofibers (Tonda-Turo et al., 2013), freeze-dried gelatin sponges (Tonda-Turo et al., 2011a) and injectable agar/gelatin gels (Tonda-Turo et al., 2014). Finally, RP methods represent advanced approaches for the preparation of complex guides with highly reproducible and controlled morphology, provided with inner coating or filler, by a single step procedure.

Biomimetic internal structures having directional cues showed improved performance in terms of regeneration even if the autologous graft remains the gold standard approach. To achieve a step forward in the field and encourage the use of artificial grafts in the clinics, multifunctional devices combining topographical, haptotactic and chemotactic cues should be investigated in-depth. Furthermore, cellularized matrices, exploiting both the paracrine and the exogenous effects, have shown preliminary promising results and have opened new possibilities in the treatment of large gap nerve injuries.

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## Figure Captions

**Figure 1**. Schematic representation of the structural features of nerve guidance channels, designed to regenerate peripheral nerves. Depicted strategies include the use of porous and not porous single lumen NGCs surface functionalised with biomimetic molecules or having micro-grooved luminal designs; intraluminal guidance structures such as fibers, sponges and gels; multi-channel NGCs to provide topographical guidance to regenerating axons and migrating Schwann cells and combinatorial approaches. Reproduced with permission from (Daly et al., 2012).

**Figure 2.** SEM micrographs of A) electrospun membrane rolled and sealed into tubes (reproduced with permission from (Chew et al., 2007)); B) electrospun PLGA/PCL nerve guide conduit (reproduced with permission from (Panseri et al., 2008)); C) bundle composed of aligned fibres (reproduced with permission from (Teo and Ramakrishna, 2005)).

**Figure 3.** SEM micrograph of A, B) PCL porous guide by Kokai et al., 2009 (reproduced with permission from (Kokai et al., 2009)); C,D) PCL porous guide by Tonda-Turo et. al, 2011a (reproduced with permission from (Tonda-Turo et al., 2011a)) ; E,F) PU60i tube (bar: A, C, E 500 µm; B, D,F 100 µm)

**Figure 4.** Percentage of FD-4 concentration in the external releasing medium respect to the initial FD-4 concentration filling the tube as a function of time, for not porous and porous PCL conduits. Data are average values ± standard deviation (n=3).

**Figure 5**. A) SEM micrograph of a fractured section of the PCL60i tube filled with GL/GP sponge. (reproduced with permission from (Tonda-Turo et al., 2011a)). B) SEM micrograph of heparan sulfate proteoglycan (HSPG)/collagen scaffolds (reproduced with permission from(Wang et al., 2012)). C,D) Perimaix nerve guides. C: SEM of the longitudinal microstructure of the Perimaix nerve guide; D: SEM of the transverse microstructure (reproduced with permission from (Bozkurt et al., 2012)).Scale bars: A 500 µm, B 100 µm, C-D 200 µm.

**Figure 6**. Scanning electron microscopy (SEM) micrographs of: A) CS/GPTMS membranes frozen at -20°C (bar: 200 µm) and B) of the fractured section of CS/GPTMS tube obtained by wrapping flat membrane (bar: 500 µm). The arrow indicates the zone where the membrane was glued to obtain the circular shape. The insert is an image of a CS/GPTMS membrane as demonstration of its flexibility.

**Figure 7**. SEM micrographs of A) cross-sectional, inner, and outer surfaces of PLGA/F127 (3 wt%) (reproduced with permission from (Oh et al., 2008)); B) PHBHV/PCL 50/50 (wt./wt.) (reproduced with permission from (Chiono et al., 2008b)). Scale bars:B-C 10 µm.

**Figure 8.** SEM micrographs of 4-channel nanofibrous PLLA scaffold (reproduced with permission from (Sun et al., 2012).

**Figure 9.** Porous CS hollow guide filled with A/GL_GP injectable hydrogels: A) image of A/GL_GP hydrogel injected into the hollow CS guide, B) SEM micrograph of freeze-dried A/GL_GP hydrogel (scale bar 100 µm).

**Figure 10.** Classification of techniques used for external guide and internal filler fabrication. Schematic representation of NGC typologies: A) hollow tube; B) flat membranes rolled and longitudinally sutured or glued into hollow guides; C) internal fillers as injectable hydrogel, porous or oriented matrices filling the guide luminal cavity; and D) bi- or multi-layered guides having one or more internal fillers covering the internal surface of the guide.

## FIGURES

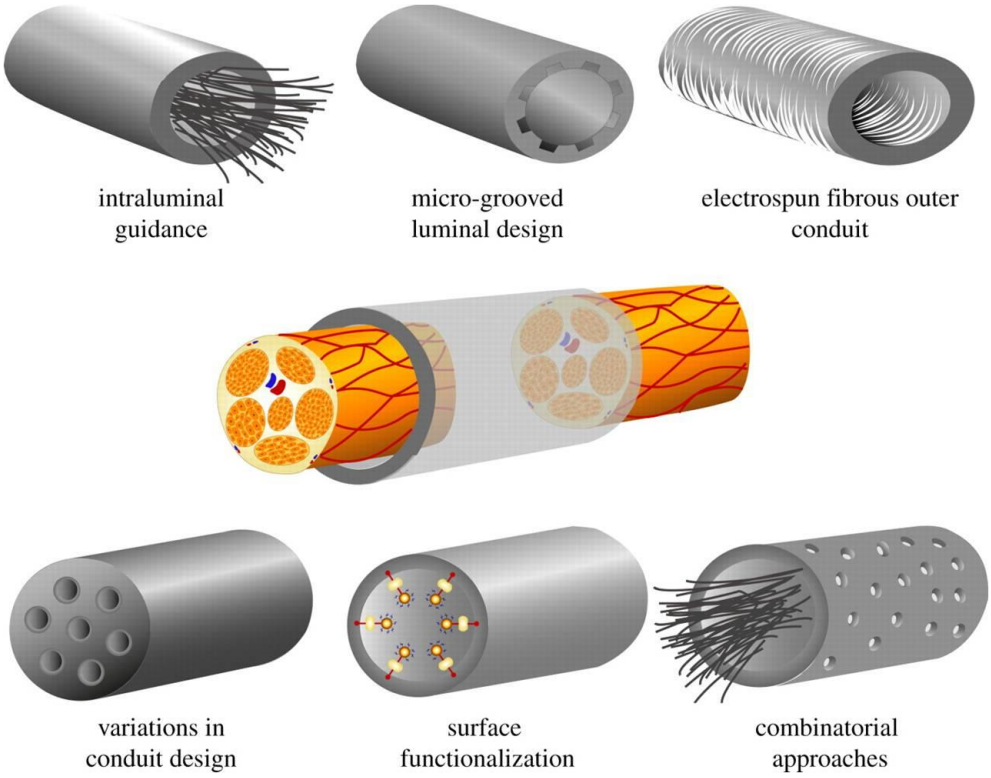



**Figure 1**

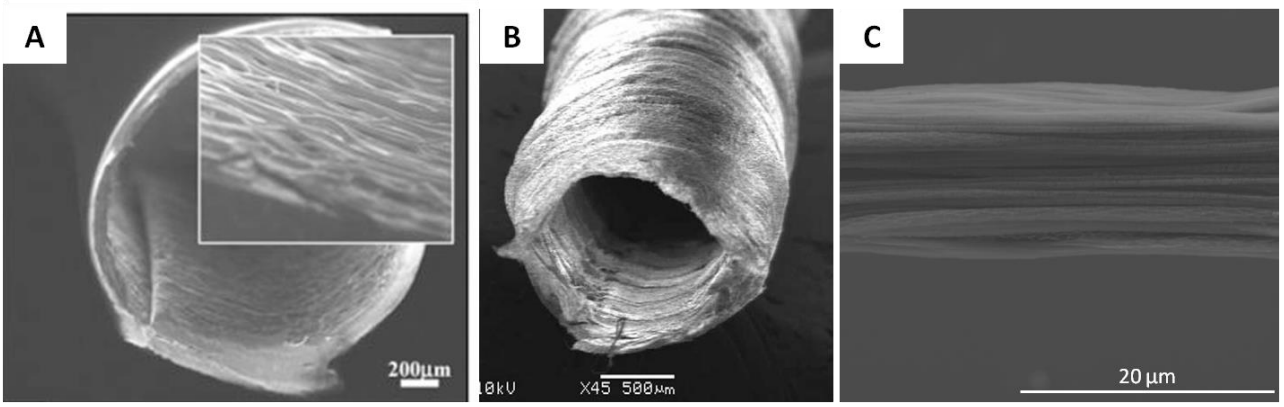



**Figure 2**

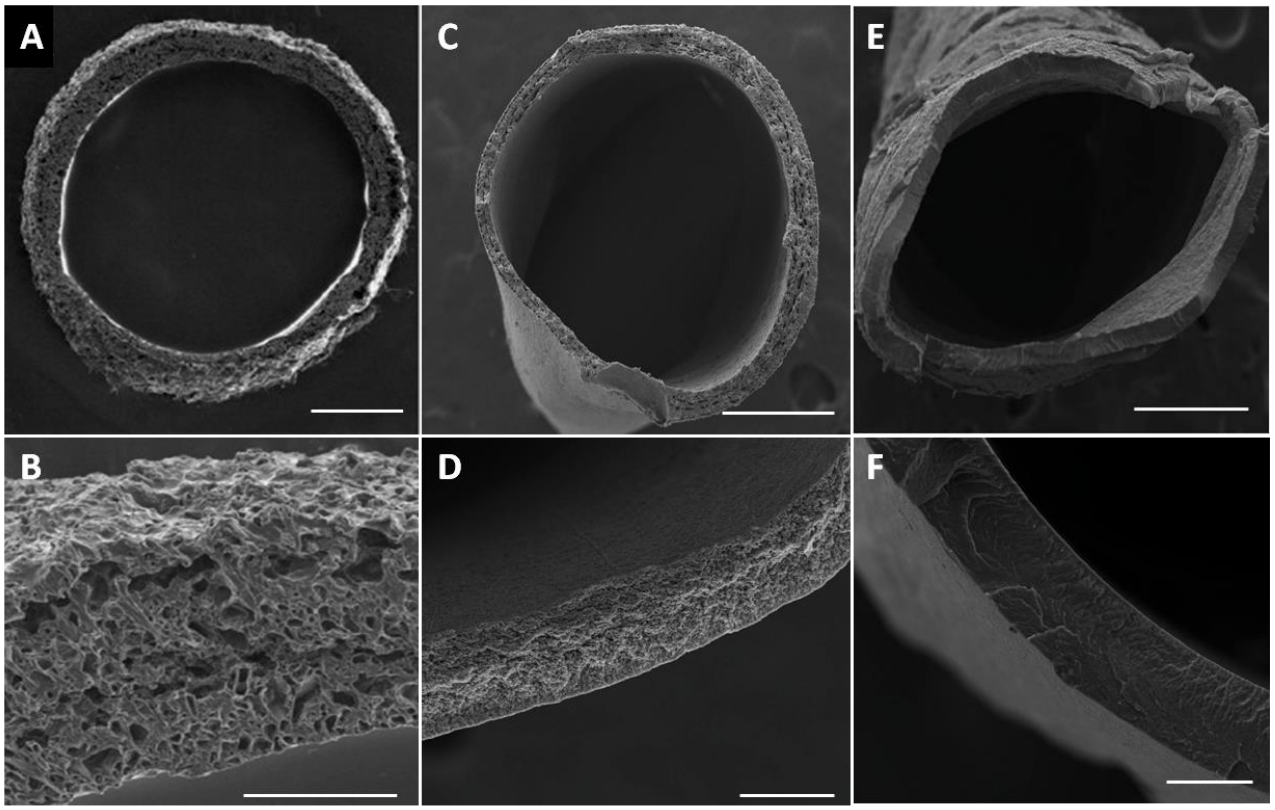
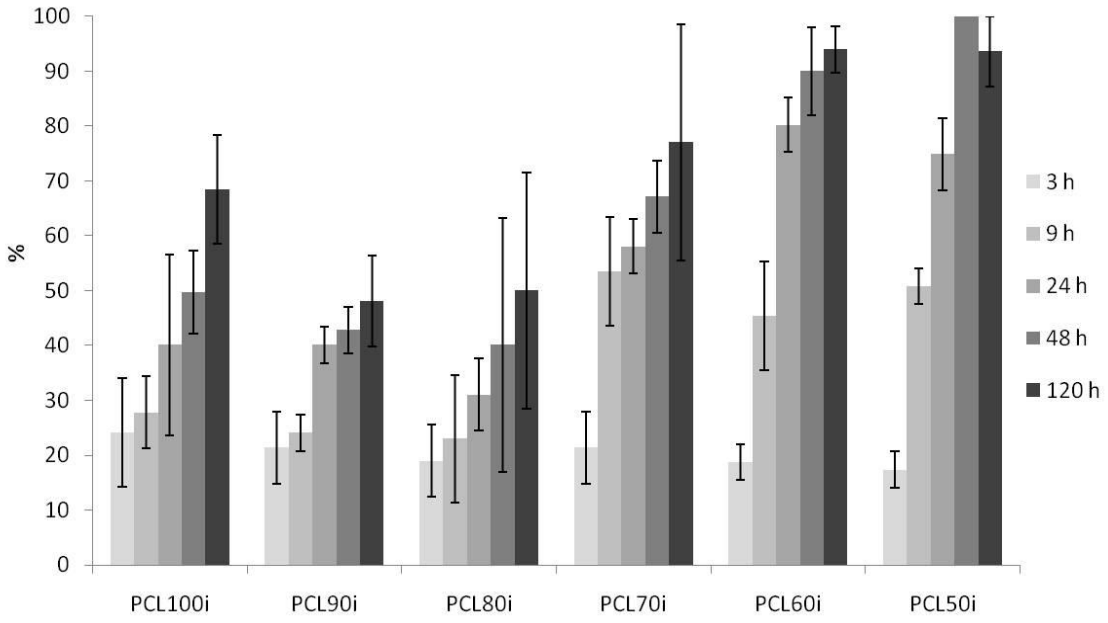



**Figure 3**

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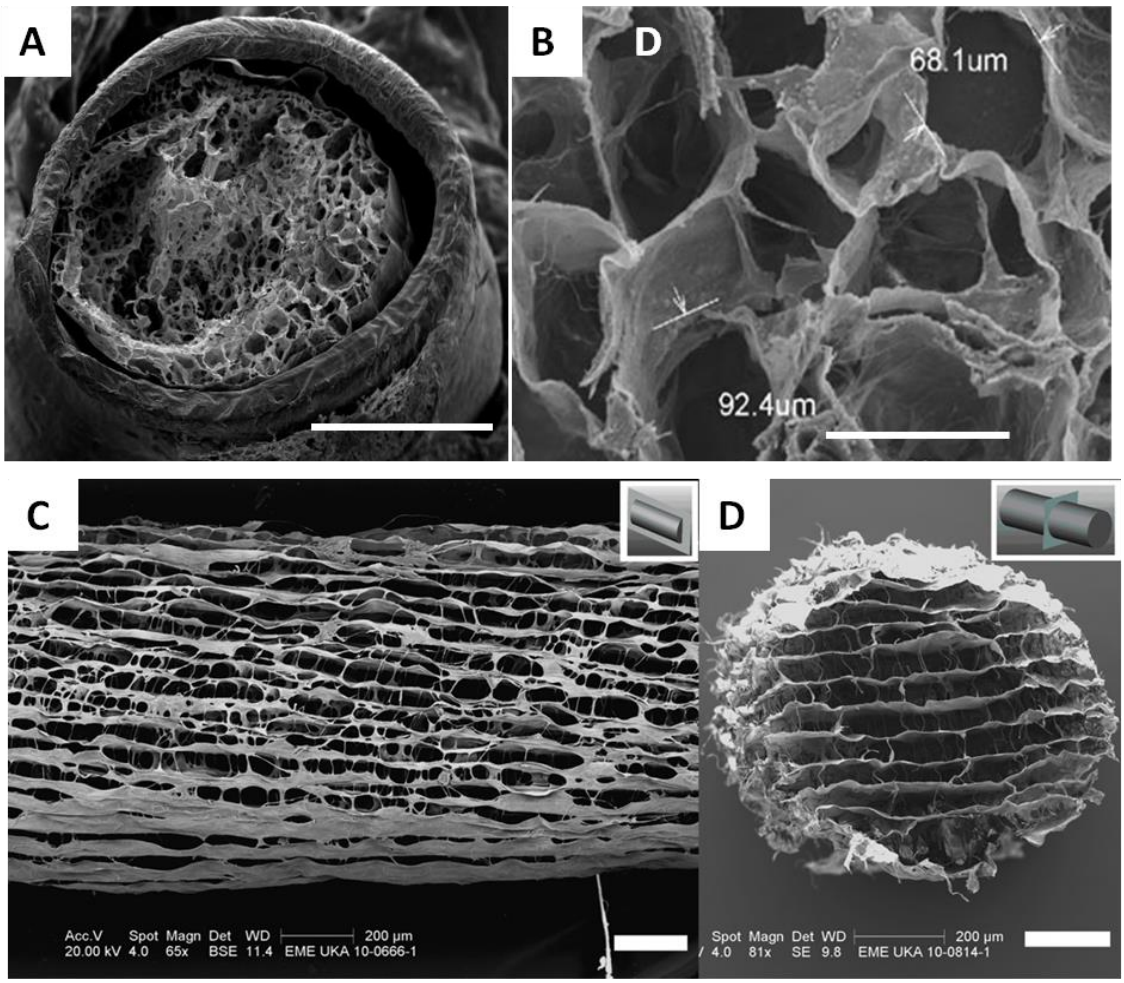


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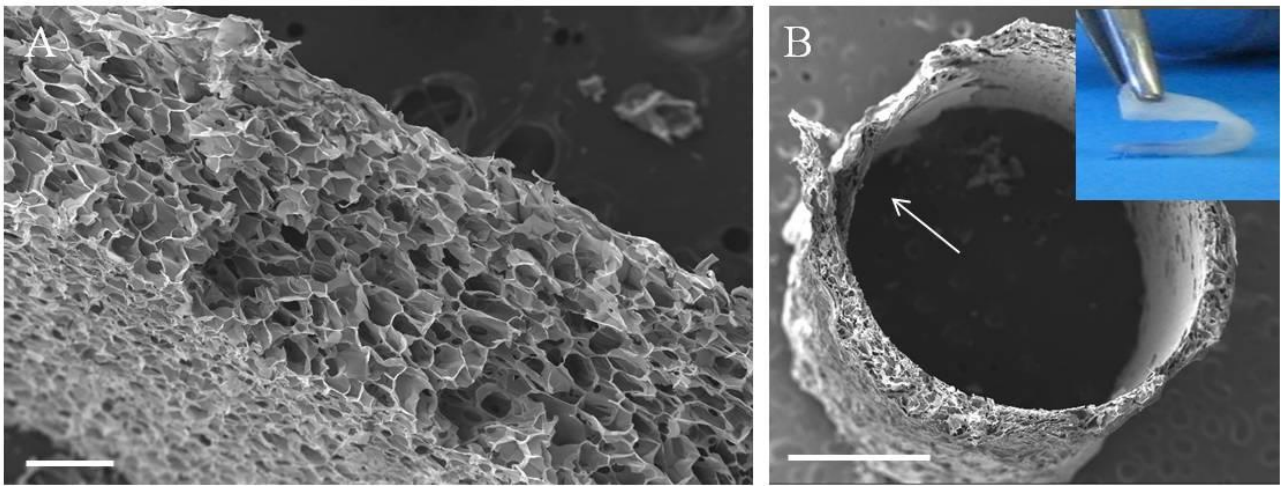


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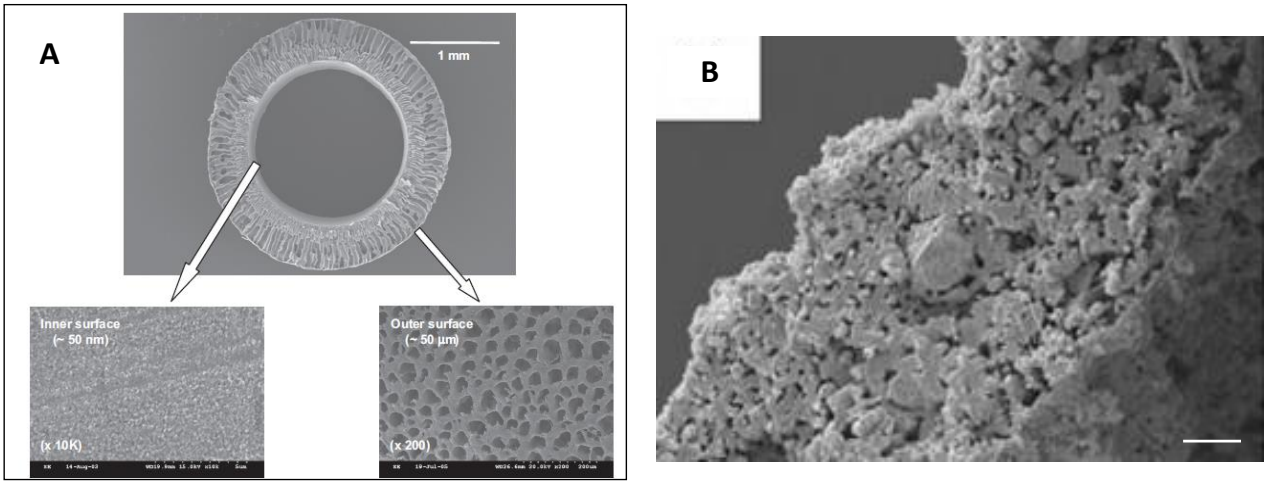


**Figure 7**

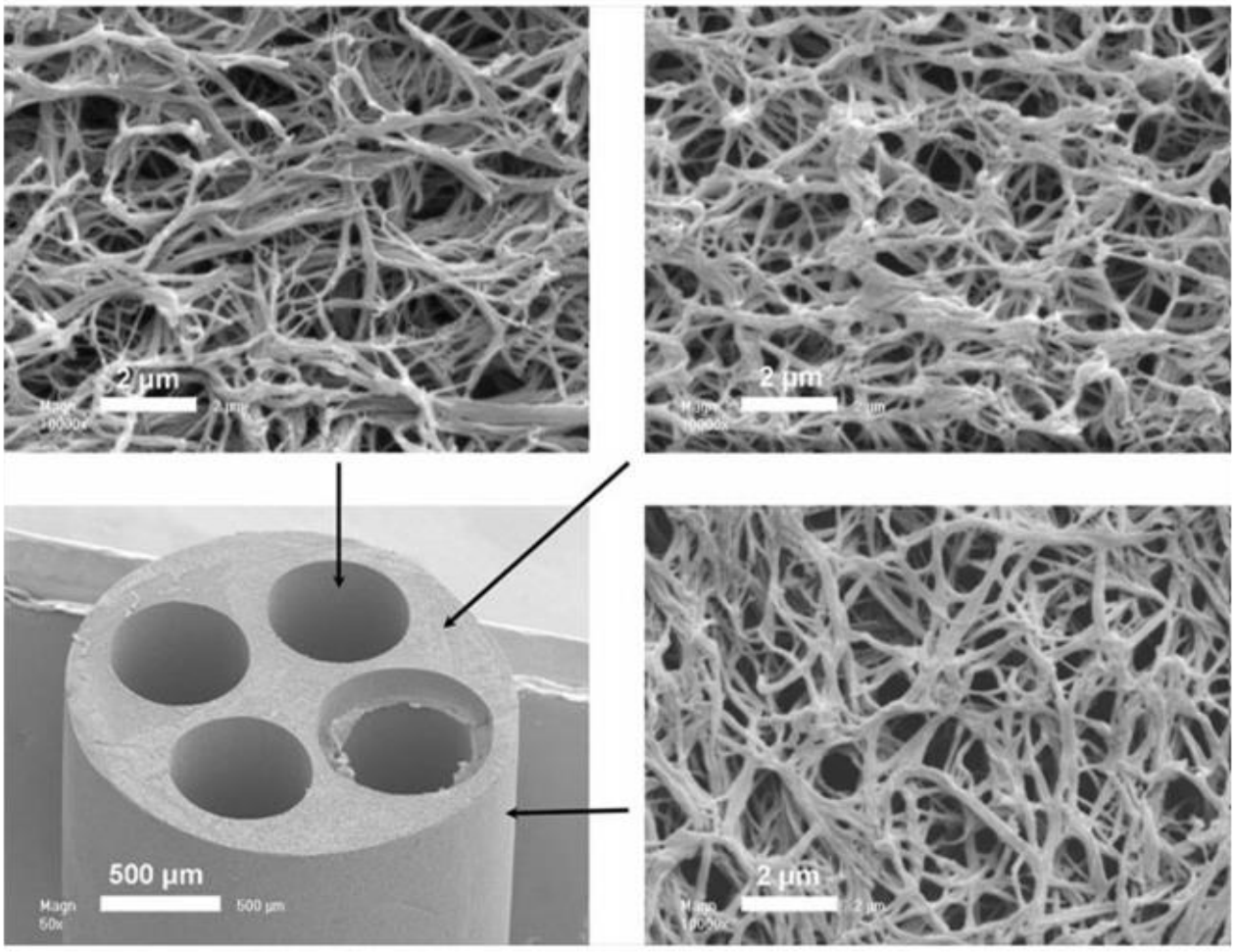


**Figure 8**

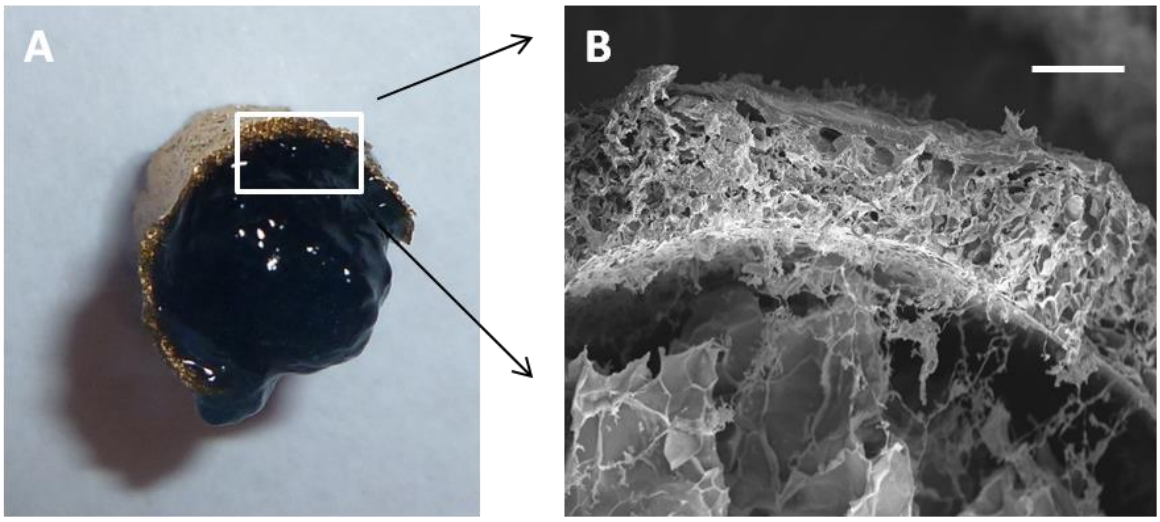


**Figure 9**

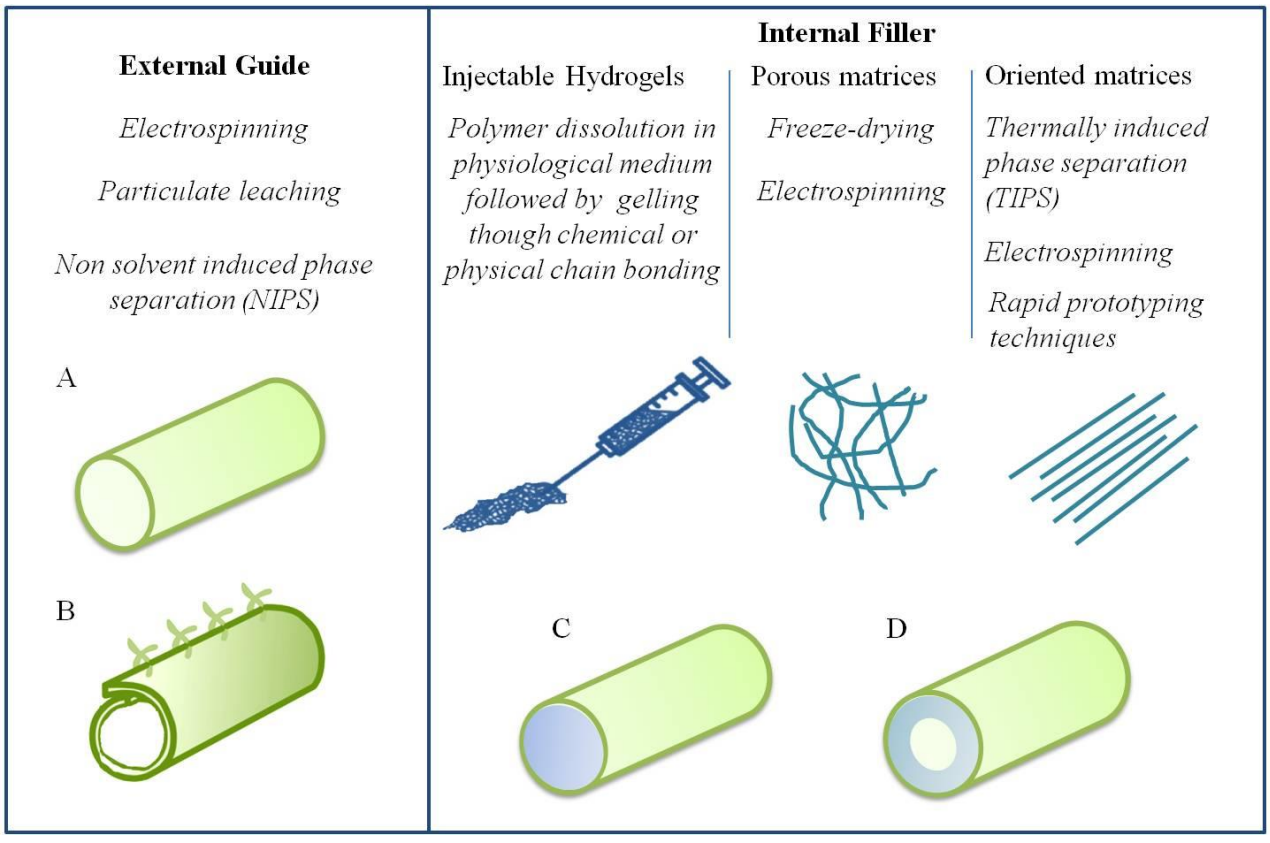


**Figure 10**

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