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Speculations on the Use of Marine Polysaccharides as Scaffolds for Artificial Nerve 'Side-'Grafts

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Abstract

Marine polysaccharides (e.g., chitosan, alginate and agarose) meet the requirements of artificial nerve grafting. These include the following: (1) prerequisites of biopolymers used as scaffolds; (2) conditions required by nerve autografts; (3) macroengineering requirements (form, design); (4) microengineering requirements (microgrooves, inclusion filaments); (5) mechanical conditions required by nerve autografts; (6) molecular aspects of peripheral nerve regeneration; space and adherence for: (i) chondroitin sulfate proteoglycans, (ii) neurite outgrowth promoting factors, (iii) neurotrophic factors, (iv) cells; (7) artificial side grafts should be compatible with autologous nerve grafts; (8) spatial distribution of neurotrophic factor gradients; (9) modulation of fibrosis; (10) renewal of luminal fillers. The mechanical stability of chitosan should be increased by adding other polymeric chains and cross-linking. As a result of deacetylation of chitin, chitosan scaffolds have got numerous positively charged free amino groups that provide adherence for growth factors and cells. To modulate fibrosis, heparin cross-linked chitosan microspheres have proved effective for delivering/transplanting cells, heparin and growth factors. To renew luminal fillers, chitosan microspheres may be injected through an indwelling catheter incorporated into the nerve conduit. Agarose and alginate have gained more acceptance as hydrogel lumen fillers. Interacting with positively charged chitosan, negatively charged alginate may form versatile chitosan-calcium-alginate microspheres.

Keywords: nerve grafting, side grafting, brachial plexus lesions, artificial nerve grafts, chitosan, agarose, alginate



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1. Introduction

After brachial plexus traction injuries, the nerve gap may be too large to be bridged by autogenous nerve grafts alone. This has prompted neuroscientists to investigate the use of artificial nerve grafts. Materials used as scaffolds for these grafts are summarized in Table 1 [1–4]. Biodegradable materials have displaced nondegradable silicone nerve conduits because these have been associated with a chronic tissue response and poor regeneration [4–6]. Several tube and sheet forms are readily available on the market for use in peripheral nerve repair [e.g., Neurotube [polyglycolic acid (PGA), Synovis], Neurolac (poly-DL-lactide-caprolactone, PLCL, Polyganics BV), NeuraGen (collagen type I, Integra NeuroSciences) and Neuro-Matrix/ Neuroflex (collagen type I, Collagen Matrix Inc) [7–10]. The degradation rate of these conduits typically ranges from months (Neurotube in 3 months and NeuroMatrix in 7 months to years; Neurolac in 16 months and NeuraGen in 4 years) [4, 11]. A few of these resorbable conduits have been tested clinically in short nerve defects [2, 12-15]. Comparable or even superior results have been reported compared to nerve autografts [13, 15, 16]. In reconstructing long nerve defects, Neurolac and NeuraGen nerve conduits have induced functional recovery similar to nerve autografts [4, 17, 18]. Nevertheless, use of nerve conduits has been limited to nerve gaps less than 3 cm in length [2, 19]. Because of this, their clinical use has been limited, especially in brachial plexus lesions.

- 1.1. Artery
- 1.2. Vein
- 1.3. Muscle
- 1.4. Tendon

2. Nonautogenous biological conduits

- 2.1. Nerve allografts
- 2.2. Decellularized biomatrices
- 2.3. Conduits consisting of basal laminae
- 2.4. Natural polymers; in vivo extracellular matrix polymers
- 2.4.1. Collagen

2.4.2. The glycosaminoglycan hyaluronic acid. Hyaluronic acid has been developed into an extremely useful copolymer

- gel with methylcellulose
- 2.5. Natural polymers; polymers derived from blood
- 2.5.1. Plasma derived polymers
- 2.5.2. Fibronectin
- 2.5.3. Fibrin
- 2.6. Natural polymers; polymers from marine life

^{1.} Autogenous biological conduits

2.6.1. Agarose is a linear polysaccharide derived from seaweed and cross-linked by temperature gradients through hydrogen bonding

2.6.2. Alginate is obtained from algae and the polymer solution is cross-linked by calcium into a sponge-like structure

2.6.3. Chitosan is a glycosaminoglycan (GAG) carbohydrate polymer derived by chemical deacetylation of chitin, the major structural polysaccharide found in crustacean, shellfish and insect shells

2.6.4. Carrageenan

2.6.5. Fucoidans

2.6.6. Ulvans

2.6.7. Hyaluronans, chondroitin sulfates, dermatan sulfates, heparan sulfates, keratin sulfates

3. Nonbiological conduits, nonabsorbable nerve conduits

3.1. Silicone

3.2. Polytetrafluoroethylene (ePTFE)

3.3. Gore-tex

3.4. Longitudinally oriented suture material (silk)

4. Nonbiological conduits, absorbable nerve conduits

4.1. Aliphatic polyesters

Members of this family include polyglycolic acid PGA, poly(lactic acid) (PLA), polycaprolactone (PCL) and their copolymers like PLGA

4.2. Poly(phosphor)esters (PPE)

Such polymers contain a characteristic phosphoester linkage in the backbone, which is more easily cleaved via hydrolysis in physiologically relevant conditions than the ester linkage. One type is poly(bis(hydroxyethyl) terephthalate-ethylphosphoester). Another one is poly(caprolactone-co-ethyl ethylene phosphate) (PCLEEP) 4.3. Polyurethanes

DegraPol is an example. It is synthesized by coupling poly(R-3-hydroxybutyric acid-co-R-3-hydroxyvaleric acid)-diol and poly(glycolide-co-caprolactone)-diol (PGCL) with 2, 2, 4-trimethylhexamethylene diisocyanate

4.4. Hydrogel-based conduits

These are based on polyethyleneglycol, a biodegradable synthetic polymer of ethylene oxide units. Hydrogel tubes are fabricated from poly(2- hydroxyethyl methacrylate) (PHEMA). PHEMA homopolymer may be copolymerized with methyl methacrylate (MMA) to provide greater rigidity. These PHEMA-MMA tubes have a biphasic wall structure, with an outer gel-like layer and inner porous layer

4.5. Piezoelectric polymers

Poled poly(vinylidene fluoride) (PVDF) conduits are fabricated by stretching PVDF conduits (to mechanically align dipoles) and then subjecting them to an electric field nA copolymer of PVDF and trifluoroethylene (PVDF–TFE) does not require mechanical stretching to align the dipoles. Positively poled PVDF–TFE conduits have shown the best results

Table 1. Scaffolds serving as nerve conduits.

The clinical use of artificial nerve grafts has gained new impetus, however. First, advances in conduit lumen filler technology have made it possible to use nerve scaffolds to bridge large peripheral nerve defects [20]. Second, after conventional end-to-end grafting, side grafting has made it possible to increase the incidence of nerve regeneration by applying additional (artificial) grafts extending from the side of the donor end to the side of the recipient end, so-called co-grafting [21]. Third, the axonal growth cone has been found to be sensitive to spatial molecular concentration gradients of nerve growth factors [22, 23]. Thus, artificial nerve side grafts supplied with spatial molecular concentration gradients of nerve growth factors might not only act as additional grafts to autografts but might provide additional stimulation to axonal growth cone progression within autografts as well. This might provide a solution for limited axonal progression in long-standing lesions (of more than one-year duration) or for partially regenerated lesions (obstetric brachial plexus lesions after 3 years of age). In light of these findings and expectations, the requirements of an artificial nerve graft have to be redefined. Marine polysaccharides (chitosan, alginate and agarose) may be modified or combined with other polymers, thus offering wide versatility to meet these requirements.

2. Requirements of an artificial nerve graft

Biopolymer scaffolds used as artificial nerve grafts should be biocompatible; their absorption and degradation kinetics should match the degree of in vivo cell/tissue growth; they should have adequate surface for cell access, proliferation and cell differentiation and lastly, they should not be toxic or carcinogenic; the same applies to their degradation products [24]. In addition, they should meet specific requirements

2.1. Artificial nerve grafts should fulfill the same conditions required by nerve autografts

In 1943, Seddon recognized three nerve injury types as follows: neuropraxia, axonotmesis and neurotmesis [25]. In 1951, Sunderland [25, 26] elaborated on this classification recognizing five degrees of nerve injury. A sixth degree has recently been added by Mackinnon [25]. Millesi [26] has subclassified Sunderland's classification according to the degree of fibrosis (**Table 1**). Autogenous nerve grafting is the standard for repair of irreducible nerve gaps (Millesi Subtypes 3C, 4N, 4S; Sunderland Type 5, Mackinnon Type 6) [26]. Autogenous grafts act as immunogenically inert scaffolds for axonal cone progression, providing simultaneously neurotrophic factors and Schwann cells for axonal regeneration [26].

2.1.1. Artificial nerve grafts should meet macroengineering requirements

To act as interposition nerve grafts, biomaterial polymer nerve scaffolds should meet macroengineering requirements, that is, they should be of proper form, design (shape) and size (diameter). In addition, they should be supplied with macrogrooves and have reasonable wall thickness. As to *form*, scaffolds may be delivered as gels, as sponges, or as solid scaffolds [10, 27]. Comparing various *designs* of porous poly(ɛ-caprolactone) synthetic polymer scaffolds (cylinder, tube, multichannel and open-path design with and without a central core), Wong et al. [28] have concluded that open path designs are superior in terms of regenerative capacity and biocompatibility (**Figure 1a** and **b**). The relationship between scaffold channel *diameter* and the number of axons regenerating has been studied. Larger areas of fibrosis and a reduced axonal numbers are associated with larger tube diameters [29]. PLGA scaffolds with multiple longitudinally aligned *macrogroove channels* of 450 and 660 μ m constructed using injection molding and seeded with Schwann cells support robust axonal growth [1, 30]. A close relationship between the formation of neuromas in regenerated tissues and the *thickness of the conduit tube wall* [31] has been observed. Tube wall thickness greater than 0.81 mm significantly attenuates axon growth [32], probably by decreasing nutrient diffusion and wall porosity. Kokai et al. have demonstrated that a wall thickness of 0.6 mm, a porosity of 80% and a pore size range of 10–40 µm are optimal for peripheral nerve repair [33, 34].



Figure 1. Nerve conduit design. According to Wong et al. [28], open path designs are preferable. It follows, that an open ³/₄ cylinder (a) is superior to a closed cylinder (b). However, both should be supplied with longitudinal macrogrooves (1) for fascicular regeneration and microgrooves (2) for axonal regeneration.

2.1.2. Artificial nerve grafts should meet microengineering requirements

Microengineering refers primarily to microgrooves directing axonal growth [35–37]. Microgrooves can be placed in the polymer surface by laser etching. A minimum groove depth of 2 μ m [38], associated with narrow ridges (5 versus 10 μ m) have improved neurite outgrowth and promoted cell adhesion [39]. Further improvements in neurite outgrowth can be obtained with coating the grooved surface with collagen or laminin peptides [40–42].



Figure 2. To enhance axonal progression, a closed cylinder nerve conduit should be filled with filaments (1), growth factors (2) and cells (3). In addition, it should be porous (4) (a). In a ³/₄ cylinder, to increase surface area to volume ratio, the wall of the cylinder should be hollow and filled with filaments, growth factors and cells (b).

Another microengineering aspect refers to axonal preference to grow along the length of micro and nanofibers of polymers [1]. The inclusion of filaments into the lumen of large tube diameter silicone tubes has been suggested by Lundborg et al. [43]. The aim has been to increase the overall cross-sectional area of the regenerated nerve fibers. Itoh et al. [44] have used eight collagen or polyester filaments. Synthetic or biological filament lumen fillers have been used ever since to enhance nerve regeneration (**Figure 2a** and **b**). Increasing the whole filament surface area by increasing their number and reducing their diameter (increased surface area-to-volume ratio) is a serious consideration [4, 43–47].

2.1.3. Artificial nerve grafts should fulfill the same mechanical conditions required by nerve autografts

A nerve conduit should possess sufficient toughness to resist compression or collapse, yet still be flexible and suturable [4, 48]. Ideally, a nerve conduit should have an elastic modulus comparable with that of a nerve autograft. In end-to-end grafting, nerve end/artificial graft elastic or shear modulus inequality might produce tension, compression or shear at the repair site, ending up with fibrosis and hampering progression of regeneration. Such inequality might also lead to rupture of the small caliber sutures (nylon or prolene 9/0 or 10/0 sutures) used for fascicular grafting. On the contrary, during side grafting, cografting with an artificial graft of slightly higher modulus of elasticity (e.g., 64.3–69.8 MPa PLLA versus 11.7 MPa peripheral nerve tensile strength [48, 49]) aids in absorbing tension off the nerve ends and off the autograft. This may improve regeneration [50, 51] (**Figures 3a–c** and **4a–c**).



Figure 3. Suturing a nerve conduit (high tensile, compression and shear modulus of elasticity) (1) in an end-to-end fashion to a nerve end (low tensile, compression and shear modulus of elasticity) (2) produces high point contact stresses at the suture lines (3), leading to their rupture (a). Also, high point contact stresses lead to an inflammatory reaction and secondary fibrosis hampering further regeneration. After successful end-to-end suturing, if a ³/₄ nerve conduit is applied as a side graft to both ends (b), it transfers 'absorbs' stresses from the sutured ends and transfers them to a whole proximal or distal segment. Unloading the suture line promotes axonal sprouting; transferring stresses to a broad segmental area decreases stresses to a point that produces some damage which is necessary for side axonal sprouting but that will not hamper axonal regeneration. The same effect is produced, when a ³/₄ nerve conduit is side grafted to an end-to-end autografted area (4) (c).

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Figure 4. Stress unloading effect of side conduits; illustrative clinical case. During a road traffic accident, a 24 year lady had sustained an intercondylar fracture of the lower end of the humerus associated with a partial injury of the ulnar nerve. The intercondylar fracture had been fixed successfully (a). The patient presented to us with partial claw hand deformity. (b) The zone of injury on surgical exploration of the ulnar nerve (area between yellow arrows). The ulnar nerve was shortened, so that the injury zone formed a U-shaped loop. The healthy proximal and distal nerve ends were next sutured to an underlying silicone sheet (green arrow) to unload the U-shaped segment (c). The patient recovered full hand function in 9 months.

2.2. Requirements based on molecular aspects of peripheral nerve regeneration

Artificial nerve scaffolds should provide adequate space for the interplay and manipulation of the different molecular pathways for axonal (peripheral nerve) regeneration [35, 36, 52, 53] (**Figure 5**).

Biomaterial polymer nerve scaffolds should provide adequate space and adherence for the following: (1) *Fibrous tissue and chondroitin sulfate proteoglycans* necessary for basal lamina synthesis; (2) *Neurite outgrowth promoting factors* [e.g., laminin (LN), fibronectin (FN), heparin sulfate proteoglycans (HSP) and tenascin]; (3) *Neurotrophic factors* which include (a) the neurotrophins [nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4/5 (NT-4/5)]; (b) the neurokines [ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF)]; (c) the transforming growth factor (TGF)-b family [TGF-b1, TGF-b2, TGF-b3, glial cell line derived neurotrophic factor (GDNF)]; (4) *Schwann cells and other cell types*. In a systematic review comparing functional results of different biomaterial polymer nerve scaffolds in rat experiments, Sinis et al. [54] have noted more favorable outcome of conduits coated with Schwann cells compared to plain synthetics.

To provide adequate space and adherence for cells and molecules, biomaterial polymer nerve scaffolds should be porous [1]. Currently, ideal scaffolding should have 80–90% porosity with a pore size of 50–250 µm. Its pores should be interconnected so as to allow for the introduction of lumen fillers [24, 55]. Lumen filler technology allows for incorporation of cells and molecular factors into nerve conduits using intermediary supporting matrices; its methods have been reviewed elsewhere (Figure 2a and b) [56]. One method involves incorporating growth factors into supporting matrices placed inside the lumen (e.g., hydrogel-forming collagen, fibrin, laminin, alginate, heparin and heparin sulfate). In general, relatively weak hydrogel-forming matrices are preferred to denser matrices because the latters can obstruct the path of axonal growth through the lumen [57, 58]. Growth factors interact with matrix materials in a number of important ways (ligand-receptor binding, ionic, electrostatic, hydrophobic, or covalent interactions). Ligand-receptor binding, for instance, may slow down the release of growth factors and protect against enzymatic degradation during nerve regeneration. A second method of lumen filling is incorporating accessory cells (e.g., Schwann cells) into a supporting matrix. Different matrices have been used successfully with Schwann cells for this purpose, such as alginate-fibronectin, collagen, gelatin and matrigel. Also, ingrowing Schwann cells and axons can be guided by such matrices for enhanced nerve regeneration. A *third* method of lumen filling is impregnating the neural conduit wall with cells or neurotrophins via cross-linking or immobilization in multichannel nerve conduits [56].



Figure 5. Molecular aspects of axonal regeneration. I. *Neurite outgrowth promoting factors* [laminin (LN), fibronectin (FN), heparin sulfate proteoglycans (HSP) and tenascin] pave the proper path by supplying orientation and adhesiveness for axons. II. *Neurotrophic factors* include a. neurotrophins (NGF, BDNF, NT-4/5); b. neurokines (CNTF, LIF); c. (TGF)-β family TGF-β1, TGF-β2, TGF-β3, GDNF. III. *Schwann cells*. All of these factors combined should be available in a nerve conduit. *The presence of single or few items (e.g., laminin, nerve growth factor, Schwann cells, mesenchymal stem cells) does not suffice for peripheral nerve regeneration*.

2.3. Requirements based on recent findings of side grafting (cografting autologous nerve grafts with biomaterial polymer nerve scaffolds)

In side grafting, the donor side is grafted to the recipient end; donor side collateral sprouting is stimulated by mechanical trauma or axotomy [25]. Similarly, the incidence of nerve regen-

eration after conventional end-to-end grafting can be increased by applying biomaterial polymer nerve side grafts extending from the side of the donor end to the side of the recipient end [21] (**Figure 3c**). Cografting autologous nerve grafts with biomaterial polymer nerve scaffolds presupposes biocompatibility between both.

2.4. Requirements based on spatial distribution of neurotrophic factor gradients

The axonal growth cone is sensitive to spatial molecular concentration gradients [22]. In a study by Rosoff et al. [22], axonal growth has been shown to be enhanced by a steep nerve growth factor (NGF) spatial concentration gradient. Axonal growth cones also preferentially advance up gradients of laminin [59]. For the spinal cord, gradients have been made in natural and synthetic polymers with laminin [1, 60]. Utilizing biomaterial polymer nerve scaffolds, axonal growth can be hypothetically made to bridge the whole length of the neural gap by seeding the scaffolds with multiple NGF spatial concentration gradients [23]. By diffusion, these NGF spatial concentration gradients might also enhance axonal growth within the adjacent natural nerve side and end-to-end autografts (**Figure 6**).



Figure 6. Spatial distribution of molecular factor gradients (1). Utilizing biomaterial polymer nerve scaffolds, axonal growth can be hypothetically made to bridge the whole length of the neural gap by seeding the scaffolds with multiple NGF spatial concentration gradients [23]. By diffusion, these NGF spatial concentration gradients might also enhance axonal growth within the adjacent natural nerve side and within the end-to-end autografts.

2.5. Requirements based on modulation of fibrosis

Scar formation prevents progression of axonal regeneration. Lysing the fibrosis/gliosis in the injury zone to an extent that allows settling of the basal lamina preventing meanwhile collapse of the neural tissue matrix is therefore essential. Recognizing this, Millesi [26] has modified Sunderland's classification of nerve injuries based on the degree of epineurial, perineurial and endoneurial fibrosis (**Table 2**). Clinically, surgical neurolysis of brachial plexus lesions has proven to be an effective procedure [50, 51, 61]. Heparin has been used for a long time in plastic surgery to lyse scar tissue [62]. Its perineural application has improved functional recovery

[63]. Among other actions, heparin influences fibroblast growth factor responsible for cell proliferation, differentiation, signal transduction and angiogenesis [64–66]. In the central nervous system, injured axons encounter a series of inhibitory factors [67]. Chondroitinase ABC lyses chondroitin sulfate proteoglycans allowing regenerating axons to progress to distal targets [68–70].

Seddon type	Sunderland type-millesi subtype
Neuropraxia	Sunderland type 1—there is transient conduction block without disruption of the anatomical structure of the nerve. Wallerian degeneration does not occur distal to the injury. Full nerve function is expected without intervention within 12 weeks
	Millesi subtype $1A-$ there is fibrosis of the epifascicular epineurium, necessitating an epineurotomy to enhance regeneration
	Millesi subtype 1B—there is fibrosis of the interfascicular epineurium, necessitating a partial epineurectomy to enhance regeneration
Axonotmesis: the axons are disrupted and Wallerian degeneration occurs	Sunderland type 2—the endoneurim and perineurium are intact and complete regeneration and functional recovery occurs.
	Millesi subtype $2A-$ there is fibrosis of the epifascicular epineurium, necessitating an epineurotomy to enhance regeneration
	Millesi subtype 2B. There is fibrosis of the interfascicular epineurium, necessitating a partial epineurectomy to enhance regeneration
	Sunderland type 3—the axon and endoneurium are damaged, but then perineurium and epineurium are intact. This results in disorganized regeneration which can result in obstruction or diversion of axons from their correct paths
	Millesi subtype 3A—there is fibrosis of epifascicular epineurium, necessitating an epineurotomy to enhance regeneration
	Millesi subtype 3B—there is fibrosis of the interfascicular epineurium, necessitating a partial epineurectomy to enhance regeneration
	Millesi subtype 3C—there is additional endoneural fibrosis. During surgery, electric stimulation of the affected nerve shows poor contraction of the muscle(s) supplied by it. Treatment is by excision of the fibrotic segment and nerve grafting
	Sunderland type 4—fourth degree injuries involve damage to all structures, however, continuity of the nerve trunk is bridged by a mass of connective tissue, Schwann cells and regenerating axons. Conduction down the nerve is not possible
	Millesi subtype 4N. The scar is invaded by neuroma. During surgery, electric stimulation of the affected nerve shows decreased or lost contraction of the muscle(s) supplied by it. Treatment is accordingly surgical by neurolysis or excision and nerve grafting
	Millesi subtype 4S—the scar consists of fibrosis only. During surgery, electric stimulation of the affected nerve shows lost contraction of the muscle(s) supplied by it. Treatment is surgical by excision and nerve grafting
Neurotmesis (complete division of	Sunderland type 5—the continuity of the nerve/brachial plexus roots, trunks, divisions or cords is completely interrupted. There may be brachial plexus root avulsions. Root avulsions are excluded by myelography or intraoperative foraminotomy. During surgery, electric stimulation of the interrupted





Figure 7. Modulation of fibrosis by heparin microspheres (1). Use can be made of both heparin as a neurolyzing agent as well as its ligand–receptor binding property. Knowing that heparin can bind FGF-2 and prevent its degradation, Han et al. have constructed a nerve conduit with a heparin-incorporated fibrin–fibronectin matrix to deliver this growth factor to the injury site [73].

Can we use artificial nerve side grafts supplied with spatial molecular concentration gradients of nerve growth factors for the following purposes simultaneously:

- to act as additional side grafts to end-to-end autografts as discussed under ' requirements based on recent findings of side grafting',

- to stimulate axonal growth cone progression within end-to-end autografts as discussed under 'requirements based on spatial distribution of neurotrophic factor gradients',

- to lyse postoperative fibrosis after brachial plexus surgery and direct cord implantation (using heparin and chondroitinase ABC respectively),

- as a result of the above, to regain full hand, elbow and shoulder functions in recent (less than 1 year) total brachial plexus palsy,

- as a result of the above, to provide a solution for limited axonal progression in long-standing brachial plexus lesions (of more than one-year duration) or for partially regenerated lesions (obstetric brachial plexus lesions after 3 years of age). This is an important consideration in view of the mounting evidence that motor endplates do not atrophy but are in a continuous regenerative process [71, 72].

Advances in lumen filling technology points to the possibility of all of the above (**Figures 2a**, **b** and 7). Use can be made of both heparin as a neurolyzing agent as well as its ligand–receptor binding property. Heparin-incorporated fibrin–fibronectin matrices can enhance nerve regeneration by binding to FGF, GDNF and NGF [73–76], preventing their enzymatic cleavage, allowing them to bind to their respective growth factor receptors with greater affinity thus improving signal transduction [77, 78] and allowing for their release at a slower rate compared to when bound to a fibrin matrix alone [79, 80].

2.6. Requirements based on the necessity of renewal (recycling) of luminal fillers to allow for the replenishment of cells, molecular factors and concentration gradients

Closely linked to the above questions, is the following question. In global brachial plexus avulsions associated with extensive fibrosis and in which the whole brachial plexus has retracted to the axilla, can we use artificial side grafts to improve the results of direct cord implantation? In such a situation, the nerve gap is extensive and associated with excessive scarring. The axonal growth cone would thus take years to reach the target muscles. Consequently, the factors mentioned before have to be replenished continually.



Figure 8. Catheter placement. Versatility is required to achieve the following three aims simultaneously: inserting and keeping the cell and drug delivery system (catheter (1)) patent; dissolving the fibrous tissue (2) that blocks pores after release of molecules and cells and replenishment of released molecules and cells associated with preservation of their spatial concentration gradients. To replenish cells and growth factors, chitosan microspheres (3) may be injected through an indwelling catheter incorporated into an external coat (4) of the nerve conduit. The internal surface of the catheter should be hydrophobic to inhibit adherence of cells and molecules to it, preventing its blockage. Although agarose and alginate have also served as nerve conduit scaffolds, they have gained more acceptance as hydrogel lumen fillers. Alginate, in particular, has been used both as a lumen filler and for encapsulation of cells and drugs. Interacting with positively charged chitosan, negatively charged alginate may form versatile chitosan-calcium-alginate microspheres. These may be useful tools for delivering cells and molecules through the catheter-conduit interface without blocking it.

This can take place through a continous cell and drug delivery system (catheter) [81, 82]. Versatility is required to achieve the following three aims simultaneously: keeping the cell and drug delivery system (catheter) patent; dissolving the fibrous tissue that blocks pores after release of molecules and cells and replenishment of released molecules and cells associated

with preservation of their spatial concentration gradients. Microsphere, nanosphere and nanoshell technology is one method to achieve the previous aims [1, 83, 84]. Controlling surface tension as well as hydrophobic and hydrophilic properties of the conduit lumen and the microspheres may help us fulfill the simultaneous three aims described previously (**Figure 8**).

3. Chitosan as a biomaterial polymer nerve scaffold

3.1. Chitosan as a biopolymer; special properties

Chitosan [poly-(β -1/4)-2-amino-2-deoxy-D-glucopyranose] [1, 24] is a *biocompatible* biopolymer made of D-glucosamine and N-acetyl-D-glucosamine bonds and β bonds (1–4), in which glucosamine is the predominant repeating unit in its structure; it is a derivative of alkaline deacetylation of chitin.

The degree of deacetylation represents the rate of D-glucosamine units with respect to the total amount of N-acetyl-D-glucosamine that makes the chitosan molecule [24]. A deacetylated chitin over 60 or 70% is already considered to be chitosan. Deacetylation transforms the acetyl group into a primary amino group, which is more hydrophilic than the preceding molecule. *The degree of deacetylation influences biodegradation*. At a greater degree of deacetylation (between 84 and 90%), the degradation process is delayed. The free amino groups in the polymer chain exposed as a result of deacetylation confer a positive charge to chitosan. The positive charge in chitosan allows for many electrostatic interactions with negatively charged molecules including nervous tissue. Chitosan tubes with 5% acetylation have been found to be most supportive for peripheral nerve regeneration [85]. Chitosan as well as its biodegradation product, chitooligosaccharide, are neurosupportive, possibly by stimulating the proliferation of Schwann cells via the miRNA-27a/FOXO1 axis [20, 86–88].

3.2. Chitosan as an artificial nerve graft scaffold fulfills the same conditions required by nerve autografts

3.2.1. Chitosan as an artificial nerve graft scaffold meets macroengineering requirements

Chitosan can be easily processed and manufactured in a variety of forms including tubes, fibers, films, sponges and hydrogels. Although nonwoven chitosan mesh conduits [89] and cell-seeded chitosan films [90] have supported axonal growth, tubes are the most common in practice, either as hollow structures or combined with lumen fillers [2, 24]. Stenberg et al. [91] have used hollow chitosan tubes for reconstruction of rat sciatic nerve defects. Gonzalez-Perez et al. [92] and others [85] have proven the regenerative capability of chitosan tubes of low (~2%) and medium (~5%) degree of acetylation, to bridge critical nerve gaps (15 mm long) in the rat sciatic nerve. Meyer et al. [93] have used fine-tuned chitosan nerve tubes with a longitudinal chitosan film as a lumen filler to reconstruct long sciatic nerve defects in rats. Nerve scaffolds composed of a chitosan nerve guidance conduit filled with polyglycolic acid (PGA)/polylactic-co-glycolic acid (PLGA) filaments have been used to bridge large peripheral nerve defects both experimentally and clinically [20].

3.2.2. Chitosan as an artificial nerve graft scaffold meets microengineering requirements

Microgrooves, microfilaments as lumen fillers and surface treatment of chitosan are microengineering procedures. *Microgrooved* chitosan or poly(DL-lactide) (PLA) conduits have been demonstrated to enhance peripheral nerve regeneration as compared to smooth conduits [94]. Zhang et al. [95] have supplied omentum-wrapped collagen-chitosan scaffolds with longitudinally oriented micro-channels to promote axonal regeneration in rats. Hu [96] has devised a nerve-guiding collagen-chitosan scaffold with inner dimensions resembling the basal lamina microchannels of normal nerves.

As far as *lumen filling* is concerned, Hu et al. [97] have demonstrated that nerve conduits filled with longitudinal aligned polyglycolic acid filaments have a better regenerative outcome for bridging large peripheral nerve gaps than hollow nerve conduits. Patel et al. [98] have blended glial cell line-derived nerve growth factor (GDNF) and laminin with chitosan to fabricate GDNF-laminin blended chitosan (GLC) nerve guides.

Microengineering applies also to *surface treatment* of chitosan to make it ready to accept molecular factors of peripheral nerve regeneration. Huang et al. [99] have investigated the surface effects of laminin modified PLGA and chitosan membranes after chemical method and plasma treatment. Results have shown that laminin has been covalently bound onto the surface of both PLGA and chitosan membranes either by chemical method or by oxygen plasma treatment. Oxygen plasma has been a better method to incorporate laminin onto the surface of membrane.

3.2.3. Chitosan as an artificial nerve graft scaffold fulfills the same mechanical conditions required by nerve autografts

Chitosan is brittle, fracturing easily under low energy [24]. Several methods have been developed to improve its mechanical properties.

In the first and most reliable one, other polymeric chains are added (e.g., polyethyleneglycol, dialdehydes). Cross-linking agents possessing at least two functional reactive groups (e.g., formaldehyde, epoxides) are used to bridge all polymeric chains together (e.g., to form glutaraldehyde and glyoxal respectively) [24]. A bio-artificial polycaprolactone (PCL)/chitosan nanofibrous scaffold has been designed and evaluated [100]. Mechanical testing has shown that Young's modulus and strain at break of the electrospun PCL/chitosan nanofibers are better than those of the chitosan nanofibers. Huang et al. [101] have used electrospun collagen-chitosan-TPU nanofibrous scaffolds The scaffolds have been cross-linked by glutaraldehyde (GTA) vapor. Wang et al. [102] have used formaldehyde-cross-linked chitosan conduits containing PGA filaments [4].

The second method makes use of the high number of positively charged amine groups consequent upon deacetylation. They are made to react with the negatively charged factors of the molecular aspects of peripheral nerve regeneration. The resulting compounds improve both the mechanical and neurotrophic aspects of chitosan. Junka et al. [103] have used nanofibrous scaffolds electrospun from blends of poly(caprolactone) (PCL) and chitosan. Taking advantage of the amine groups on chitosan, the surface of the scaffolds have been functionalized with laminin by carbodiimide based cross-linking. Wang et al. [102] have compared chitosan nano/microfibrous tubes with a deacetylation rates of 78 and 93% to each other. Chitosan nano/microfiber mesh tubes with a deacetylation rate of 93% have had sufficient mechanical properties to preserve tube space and to provide a better scaffold for cell migration and attachment. Haastert-Talini et al. [85] have compared chitosan tubes of varying degrees of acetylation (low-2%, medium-5%, high-20%) for bridging peripheral nerve defects. Chitosan tubes with 5% acetylation have been found to be most supportive for peripheral nerve regeneration.

The third method consists of coating chitosan tubes with a film composed of a material of high mechanical toughness. Duda et al. [104] have reported on composite nerve grafts with an inner 3D multichannel porous chitosan core and an outer electrospun polycaprolactone shell. Also, cellulosic fibers in nano scale have been found to be an attractive reinforcement to chitosan, producing environmental friendly composite films with improved physical properties [105].

In the fourth method, the scaffold shape is additionally varied. Itoh et al. [106, 107] have developed nerve scaffolds using tendon chitosan tubes or hydroxyapatite-coated tendon chitosan tubes having either a circular or triangular cross-section, as well as triangular tubes combined with laminin. The mechanical strength of triangular tubes has been found to be higher than circular tubes and the inner volume of a triangular tube tends to be larger than in circular tubes. Nerve tissue regeneration along the tube wall has been present in both the laminin and laminin peptide groups.

In the fifth method, lumen fillers have been used. Xue et al. [108] have used a neural scaffold that consisted of a chitosan conduit inserted with poly(lactic-co-glycolic acid) (PLGA) fibers and mesenchymal stem cells to bridge an extra-large gap in dog sciatic nerve.

3.3. Chitosan as an artificial nerve graft scaffold fulfills requirements based on molecular aspects of peripheral nerve regeneration; chitosan fulfills requirements based on spatial distribution of neurotrophic factor gradients

The interaction of chitosan and molecular aspects of peripheral nerve regeneration obeys principles of luminal filling. The principles of lumen filling can also be applied to create spatial concentration gradients of molecular factors. Three main factors determine this: the degree of deacetylation of chitosan, its porosity and chitosan biodegradation products (chitooligosac-charides).

Impregnating the *neural conduit wall* with *cells* or *neurotrophins* via cross-linking or immobilization is one way of lumen filling [56]. *Cell adhesion* to the structure is determined by the extent of its positive charge, itself a function of the degree of alkaline deacetylation [1, 24]. This effect has been reported for a number of anchorage-dependent cells, such as dorsal root ganglion neurons, Schwann cells [86], neural stem cells [109] and mesenchymal stem cells [110–112]. Examples for impregnating the nerve conduit wall with *neurite outgrowth promoting factors and neurotrophins* include the use of laminin [113, 114], neurotrophin-3 [115] and transforming growth factor- β 1 [116].

Combining growth factors with a lumen growth-supporting matrix; incorporating accessory cells into the lumen matrix; seeding (genetically engineered) cells that produce growth factors inside the lumen; and/or using microspheres to deliver growth factors or accessory cells to the NC lumen are four other methods of luminal filling [56]. The latter four methods require pores. Porous chitosan scaffolds contain pores with size ranges from 1 to 250 µm and that vary according to temperature and water content. The lower the temperature and the greater the water content, the smaller the pore size. The porous structure can be stabilized by adding glutaraldehyde, polyethyleneglycol, heparin, or collagen, allowing the structure to become more resistant and to maintain elasticity[24]. However, the need for cross-linking agents may be eliminated [20, 117, 118]. A natural and low-toxicity cross-linking agent, genipin, has been used to immobilize nerve growth factor (NGF) onto chitosan-based neural scaffolds [119].

Lumen matrix biomaterials include collagen [120], fibronectin or hydrogel [121]. All bind molecular factors and cells with high affinity and release them slowly over time [56].

The biodegradation products of chitosan are water-dissolvable chitooligosaccharides (COSs), which have been shown to support peripheral nerve regeneration. Jiang et al. [122] have demonstrated that the beneficial effect of *chitooligosaccharides* on cell behavior and function of primary Schwann cells is accompanied by up-regulation of adhesion proteins and neurotrophins.

3.4. Chitosan as an artificial nerve graft scaffold fulfills requirements based on recent findings of side grafting

Cografting autologous nerve grafts with biomaterial polymer nerve scaffolds presupposes biocompatibility between both. Evidence for neural cell/chitosan biocompatibility comes from an animal model of multiple sclerosis developed by Hoveizi et al. [123]. These authors have noted the role of polylactic acid/chitosan (PLA/CS) scaffold in increasing neural cell differentiation. The biocompatibility of functional Schwann cells induced from bone mesenchymal cells with a chitosan conduit membrane has been proven by Zhang et al. [124].

3.5. Chitosan as an artificial nerve graft scaffold fulfills requirements based on modulation of fibrosis

Firstly, through its cationic interactions, chitosan prevents secondary infection by many types of fungi, yeasts and bacteria [24]. Secondly, chitosan exerts an antifibrotic effect by binding to collagen via hydrogen bonding and polyanionic–polycationic interactions. Thus it prevents postoperative abdominal adhesions either singly, or as a polypropylene/chitosan mesh, or even as a chitosan-gelatin modified film. Experimentally, it has been used to prevent excessive fibrosis after intestinal resection anastomosis. The feasibility of using silk fibroin and chitosan blend scaffolds for preventing excessive fibrosis after ventral hernia repair has been investigated in guinea pigs [24].

Thirdly, heparin, both an anticoagulant and fibrotic drug, can be incorporated into chitosan microspheres or can be used as a luminal filling matrix molecule in chitosan conduits. Heparin

cross-linked chitosan microspheres for the delivery of neural stem cells and growth factors for central nervous system repair have been used by Skop et al. [125]. This has proved effective for transplanting large numbers of neural stem cells, heparin and FGF-2. Using chitosan nerve conduits, Han et al. [73] have developed a basic fibroblast growth factor delivery system fabricated with heparin-incorporated fibrin-fibronectin matrices for repairing rat sciatic nerve disruptions.

3.6. Requirements based on the necessity of renewal (recycling) of luminal fillers to allow for the replenishment of cells, molecular factors and concentration gradients

Chitosan offers the versatility required in microsphere, nanosphere and nanoshell technology [1, 91] as one method to achieve three aims simultaneously: dissolving the fibrous tissue that blocks pores after release of molecules and cells as aforementioned, replenishment of released molecules and cells associated with preservation of their spatial concentration gradients and keeping the cell and drug delivery system (catheter) patent.

To replenish released molecules and cells, Zeng et al. [10] have incorporated chitosan microspheres into collagen-chitosan scaffolds for the controlled release of nerve growth factor. Wei et al. [126] have evaluated the potential of a chitosan/silk fibroin scaffold serving as a delivery vehicle for adipose-derived stem cells in neural tissue. Wang et al. [127] have used Schwann cells on oriented chitosan nanofiber mesh tubes as delivery vehicles. Maysinger et al. [128] have prepared microspheres containing ciliary neurotrophic factor (CNTF) or genetically engineered cells able to synthesize and release this cytokine. Raisi et al. [129] have resorted to mesenchymal stem cell-derived microvesicles to enhance sciatic nerve regeneration in the rat.

Keeping the cell and drug delivery system (catheter) patent may necessitate external coating of the nerve conduit [104]. Part of the external coating may be made of a hydrophobic material, preventing microsphere adherence to it and thus keeping it patent. Electrically and magnetically responsive microspheres may be exposed to repulsive fields in hydrophobic areas. Indeed these microspheres have been used to enhance nerve regeneration. Qi et al. [130] have electrically stimulated olfactory ensheathing cells (OECs) using a biodegradable conductive composite made of conductive polypyrrole (PPy, 2.5%) and biodegradable chitosan (97.5%). Similarly, Liu et al. [131] have activated Schwann cells in vitro using magnetically responsive magnetic nanoparticles (MNPs) and a biodegradable chitosan-glycerophosphate polymer.

4. Other biomaterial polymer nerve scaffolds

Although agarose and alginate have also served as nerve conduit scaffolds, they have gained more acceptance as hydrogel lumen fillers. Alginate, in particular has been used both as a lumen filler and for encapsulation of cells and drugs.

4.1. Agarose

4.1.1. Agarose as a biopolymer; general properties

Agarose consists of alternating units of b-D-galactopyranose and 3, 6-anhydro-a- L-galactopyranose; extracted from different sources it can have different chemical compositions depending on Sulphated L-hydroxyl group concentrations [1, 132]. Agarose is thermoresponsive, but poorly degradable

4.1.2. Agarose as a nerve scaffold

4.1.2.1. Macroengineering and microengineering requirements

Stokols and colleagues have developed agarose-based scaffolds with linear pores by using a freeze-drying process [133] as well as by templating [134]. These authors have used a freeze drying method to form agarose scaffolds containing linear guidance pores with a mean diameter of 120 μ m [133, 135]. This process involves the formation of ice crystals whose size and direction of growth can be controlled by the temperature gradient [136]. Pore size in the scaffold can also be controlled by the freezing rate and pH; the faster rate the smaller the size [137]. Integrating BDNF into the scaffold material or using BDNF-secreting mesenchymal stem cells scaffold channels, has significantly improved the scaffold's capacity to promote regeneration [135]. Koffler et al. [138] have implanted linear-channeled agarose scaffolds in spinal cord injuries, providing growing axons with linear growth paths at the lesion site and minimizing regeneration distance. The linear channels have helped regenerating axons maintain the correct mediolateral and dorsoventral position in the spinal cord.

4.1.2.2. Pore size and mechanical properties

The average pore size of agarose hydrogels is influenced by the concentration of the polymer in solution and the settling temperature. An increase in concentration results in tightly packed helices that translate to a decrease in pore size. Decreasing the settling temperature results in small pore gels with higher elastic moduli [132].

4.1.2.3. Molecular aspects of peripheral nerve regeneration

The interaction of agarose and molecular aspects of peripheral nerve regeneration obeys principles of luminal filling. Agarose does not provide adhesion motifs to cells and does not allow interaction between adherent cells and the entrapping matrix. However, it can be supplemented with adhesion molecules of the extracellular matrix [56, 139, 140].

In repairing injuries in the CNS, agarose-based biomaterials have also had incredible success, at least in experimental settings [133, 134]. BDNF within lipid microtubules has been incorporated into agarose scaffolds, enhancing axonal growth for the length of the scaffold but not into the distal cord [70]. Living neurons and axonal tracts have been internalized within agarose hydrogel tubes by Cullen et al. [141]. In a study by Gros et al. [142], templated agarose scaffolds have substantially enhanced the organization and distance over which long-tract

axons extend through a spinal cord lesion site in the presence of combinatorial therapies, but host-scaffold reactive interfaces have limited axon re-penetration of the host.

4.1.3. Agarose as a scaffold lumen filler

In order to promote sciatic nerve regeneration across a challenging 20 mm nerve gap in rats, Dodla and Bellamkonda [140] have used anisotropic scaffold lumen fillers of agarose hydrogels containing gradients of laminin-1 (LN-1) and nerve growth factor (NGF) molecules. Injectable agarose hydrogels have also been implemented [70, 143]. Using multi-photon chemistry, Wylie et al. [144] have created 3D agarose hydrogels that incorporate two bioactive molecules (a transcription factor and a growth factor).

4.2. Alginate

4.2.1. Alginate as a biopolymer; general properties

Composed of (1–4)-linked α -L-guluronate (G) and β -D-mannuronate (M) monomers, alginat*e* has carboxyl groups which are charged at pH values higher than 3–4, making it soluble in neutral and alkaline media [145]. Alginate is biocompatible, has low toxicity and high bioa-vailability [132]. Alginates can form polyelectrolyte complexes in the presence of polycations such as poly-L-lysine or chitosan. Poly-L-lysine has been widely used to coat alginate beads [132].

4.1.2. Alginate as a nerve scaffold

4.1.2.1. Macroengineering and microengineering requirements

Anionic in nature, alginate can be assembled with polycations into polyelectrolyte freestanding films (i.e., films with a few micrometers in thickness) which are suitable drug reservoirs of biomolecules, such as growth factors and antibiotics [145]. Lu et al. [146] have reported on the use of chitin-alginate 3D microfibrous scaffolds to support efficient neuronal differentiation and maturation under chemically defined culture conditions. Ethylene diamine has been covalently cross-linked to further freeze-dried alginate, resulting in a porous foam. This has been subsequently implanted into a 7-mm sciatic nerve gap in the rat without suturing [147]. Alginate foams have also been tested alone or in combination with a protective PGA mesh in the treatment of a 50-mm sciatic nerve defect in the cat [148]. Alginate foams can promote nerve regeneration even without suturing to the nerve stumps, or enclosing within a tube structure [4].

4.1.2.2. Molecular aspects of peripheral nerve regeneration

Wnt proteins are bifunctional axon guidance molecules, several of which appear to mediate guidance of corticospinal tract axons along the spinal cord. Park et al. [149] have studied the effect on spinal cord regeneration by Wnt-containing alginate scaffolds. In another study

[56, 150], nerve growth factor (NGF) has been cross-linked to the wall of an alginate/chitosan scaffold.

4.1.2.3. Modulation of fibrosis

Heparin can be used both as a neurolyzing agent and as a matrix lumen filling ligand-binder. A heparin/alginate gel combined with basic fibroblast growth factor but without a tubular structure [151] has been used as an artificial nerve graft for digital nerve repair. A matrix consisting of heparin and alginate covalently cross-linked with ethylenediamine has been produced to stabilize and control the release of growth factors [152].

4.2.3. Alginate as a scaffold lumen filler

4.2.3.1. Hydrogel matrix as lumen filler

Alginate hydrogels have been used as lumen fillers in association with *growth factors* such as LIF, a pleiotrophic protein [74, 153], fibroblastic growth factor 2 (FGF2) [154] and glial derived growth factor (GGF) [155, 156]. Alginate hydrogels have been used as lumen fillers in association with *cells*, such as Schwann cells [58, 157], allogenic Schwann cells [158] and olfactory ensheathing cells (OECs), Schwann cells (SCs) and bone marrow stromal cells (BMSCs) [159].

4.2.3.2. Encapsulated particles as lumen fillers

Cells and growth factors may be encapsulated in protective alginate microcapsules, coated with multilayers of chitosan and alginate. Poly(lactic acid) microparticles may be co-encapsulated to provide anchorage points thus promoting cell survival [145]. Alginate encapsulation has involved, among other things, the encapsulation of olfactory ensheathing cells (OECs) [160], fibroblasts engineered to produce brain-derived neurotrophic factor (Fb/BDNF) [161] and NGF-expressing cells [162].

5. Conclusion

We have outlined the requirements of artificial nerve end-to-end or preferably side grafting. These include: general prerequisites of biopolymers used as scaffolds; conditions required by nerve autografting; macroengineering requirements; microengineering requirements; mechanical conditions required by nerve autografting; requirements based on molecular aspects of peripheral nerve regeneration; requirements of side grafting; requirements based on spatial distribution of neurotrophic factor gradients; requirements based on modulation of fibrosis; requirements based on the necessity of renewal of luminal fillers to allow the replenishment of cells, molecular factors and concentration gradients. We have also outlined that using nerve conduit as side grafts necessitates their design as open cylinders, so they can be easily placed around suture lines and nerve graft and so that to allow diffusion of nutrients to the autografts. Side-grafting unloads stresses from autografts and nerve ends. They allow of spreading nerve

growth factor concentration gradients and dissolution of fibrosis. Chitosan satisfies all these requirements. Combined with other materials, it makes them less stiff, thus mechanically suitable as grafts. Produced as a result of deacetylation of chitin, chitosan nerve conduit scaffolds have got numerous positively charged free amino groups, which enable them to provide adherence for nerve growth factors and cells by lumen filling methods. To modulate fibrosis, heparin cross-linked chitosan microspheres have proved effective for delivering/ transplanting large numbers of neural stem cells, heparin (matrix molecule and neurolyzing agent) and FGF-2. To replenish cells and growth factors, chitosan microspheres may be injected through an indwelling catheter incorporated into an external coat of the nerve conduit. The internal surface of the catheter should be hydrophobic to inhibit adherence of cells and molecules to it, preventing its blockage. Polystyrene can be used to create superhydrophobic surfaces. The real challenge remains the catheter-conduit interface. This cannot be kept hydrophobic; nevertheless, it should be kept patent. Agarose and alginate have mainly served as hydrogel lumen fillers. Alginate, in particular, has been used both as a lumen filler and for encapsulation of cells and drug. Interacting with positively charged chitosan, negatively charged alginate may form versatile chitosan-calcium-alginate microspheres. Both cationic chitosan- and anionic alginate-coated poly(DL-lactide-co-glycolide) nanoparticles [163] and chitosan/alginate beads [164] have been produced and may be useful tools for delivering cells and molecules through the catheter-conduit interface without blocking it.

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