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# Novel systems for tailored neurotrophic factor release based on hydrogel and resorbable glass hollow fibers

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ABSTRACT

A novel system for the release of neurotrophic factor into a nerve guidance channel (NGC) based on resorbable phosphate glass hollow fibers ($50P_2O_5$–$30CaO$–$9Na_2O$–$3SiO_2$–$3MgO$–$2.5K_2O$–$2.5TiO_2$ mol%) in combination with a genipin-crosslinked agar/gelatin hydrogel (A/G_GP) is proposed.
No negative effect on the growth of neonatal olfactory bulb ensheathing cell line (NOBEC) as well as on the expression of pro- and anti-apoptotic proteins was measured in vitro in the presence of fiber dissolution products in the culture medium. For the release studies, fluorescein isothiocyanate–dextran (FD-20), taken as growth factor model molecule, was solubilized in different media and introduced into the fiber lumen exploiting the capillary action. The fibers were filled with i) FD-20/phosphate buffered saline (PBS) solution, ii) FD-20/hydrogel solution before gelation and iii) hydrogel before gelation, subsequently lyophilized and then filled with the FD-20/PBS solution. The different strategies used for the loading of the FD-20 into the fibers resulted in different release kinetics. A slower release was observed with the use of A/G_GP hydrogel. At last, poly(ε-caprolactone) (PCL) nerve guides containing the hollow fibers and the hydrogel have been fabricated.



## 1. Introduction

The peripheral nerve axons can spontaneously regenerate after nerve injury thanks to the ability of the Schwann cells to promote a permissible environment for axonal growth [1]. In spite of this spontaneous regeneration, a complete recovery of nerve function after a severe lesion is unlikely to occur and clinical results have been, so far, unsatisfactory [2].

After peripheral nerve injuries, the capability of injured axons to regenerate and recover functional connections depends on the type of lesion and the distance over which the regenerating axons must grow to reinnervate their peripheral targets. After nerve crush, regeneration is usually successful, since the continuity of the endoneurial tubes is preserved [3].

In the case of more severe injuries, involving damages of the perineurium and endoneurium or the complete nerve transection, the spontaneous regeneration process is compromised and a surgical intervention becomes necessary [4]. For large nerve defects, in which direct suture of the two stumps (i.e., end-to-end suture) would generate excessive tension, the bridging of the gaps with an autologous nerve graft is currently considered the current gold standard [4,5]. The use of a nerve guidance channel (NGC) sutured in between the two nerve stumps represents an alternative to autograft implantation, avoiding the additional surgical procedure needed for nerve graft harvesting. Moreover, NGC implies a less surgical trauma due to fewer epineurial sutures, the absence of any interference from the imperfectly aligned autograft fascicles, the minimization of fibrous scar tissue infiltration and the maximization of soluble factor accumulation [5].

Some NGCs are currently available on the market [4,6,7], but their use is limited to short nerve defects, up to about 3 cm [8]. For large nerve gaps, an inadequate formation of the fibrin cables between the two nerve stumps limits the migration of native SCs and the formation of bands of Büngner, which are the trophic and topographical guidance structures for the regenerating axons growing from the proximal stump. Another important issue can be an insufficient neurotrophic support into the NGC [9]. For these reasons, there is a need to confer additional functionalities to the NGCs in order to improve the regeneration of longer nerve defects. A number of neurotrophic factors have been shown to stimulate axonal regeneration when placed in the lumen of the tube. These include the nerve growth factor (NGF) [10], fibroblast growth factor (FGF) [11], vascular endothelial growth factor (VEGF) [12], insulin-like growth factor-I (IGF-I) [13] and a combination of the platelet-derived growth factor BB (PDGF-BB) and IGF-I [14]. Growth factors (GFs) are usually delivered via a carrier because of their limited activity in vivo; for instance, VEGF has a clearance half-life of less than 1 h following injection in vivo [15]. Therefore, systems guaranteeing the stability and a controlled release of a GF are required in order to mimic the temporal presence of GFs in the biological environment.

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In a previous study, Vitale-Brovarone et al. [16] showed that phosphate glass fibers are able to support glial and neuronal cell adhesion and to direct the growth of long axons, showing to be promising for the creation of topographical guidance structures for cells during nerve regeneration. Moreover phosphate glass hollow fibers could easily incorporate a liquid exploiting the capillary action and subsequently release it [17]. In this study, phosphate glass hollow fibers are proposed as a means for the incorporation and release of neurotrophic factors into a NGC. Fluorescein isothiocyanate–dextran (FD-20) is selected as a model molecule to study the release of biomolecules from the glass fibers. FD-20 is a fluorescent molecule having a molecular weight (Mw) around 22 kDa and a Stokes radius of 33 Å (supplier's data) comparable to the dimensions of many relevant GFs in nerve regeneration (e.g., NGF, VEGF, brain-derived neurotrophic factor—BDNF, glial cell-derived neurotrophic factor—GDNF, all having molecular weights in the range of 20–30 kDa as dimers) [18].

In order to tailor the release kinetics from the glass hollow fibers, an agar (A)/gelatin (G) hydrogel crosslinked with genipin (A/G_GP) is proposed as a filler for the hollow fibers. This hydrogel was previously described by Tonda-Turo et al. [19] as an injectable filler for NGCs. The mild conditions for hydrogel preparation (low temperature and physiological pH) are advantageous for the incorporation of bioactive molecules avoiding their denaturation, while hydrogel microporosity allows an efficient biomolecule delivery.

Finally, a multifunctional NGC was fabricated filling a porous poly(ε-caprolactone) (PCL) guide with both glass hollow fibers and A/G_GP hydrogel.

## 2. Materials and methods

### 2.1. Hollow fiber drawing

Phosphate glass hollow fibers (glass composition $50P_2O_5$–30CaO–$9Na_2O$–$3SiO_2$–$3MgO$–$2.5K_2O$–$2.5TiO_2$ mol%, coded as $TiPS_{2.5}$ [20,21]) were drawn using an in-house developed drawing tower equipped with an online optical fiber diameter monitor, as previously described [16]. In brief, the precursors of the glass (Sigma-Aldrich) were melted in a 90Pt/10Rh wt.% crucible in a furnace and cast into a preheated (below 440 °C, which is the glass transition temperature) stainless steel mold, subsequently rotated around its longitudinal axis (3400 rpm) using an homemade rotational equipment (no load motor, 50 W). A cylindrical glass hollow preform (outer and inner diameters, $D_{po} = 11.3$ mm and $D_{pi} = 6.5$ mm, respectively) was obtained and then annealed (410 °C, 15 h). The preform was heated at 610 °C in the drawing tower causing the formation of a neck-down region with the creation of a thin fiber which was collected on a rotating drum. Acting on the preform feeding speed, $v_p$, and the fiber speed, $v_f$, it was possible to obtain two types of fibers (coded as FA and FB) with different diameters (Table 1). The theoretical fiber outer ($TD_{fo}$) and inner ($TD_{fi}$) diameters were calculated imposing the mass conservation law and the retention of the outer to inner diameter ratio of the hollow preform ($R = D_{fo}/D_{fi} = D_{po}/D_{pi} = 1.74$) using the following formulas:

$$TD_{fo} = D_{po}\sqrt{\left(v_p/v_f\right)} \quad (1)$$

$$TD_{fi} = TD_{fo}/R. \quad (2)$$

**Table 1**
Preform feeding speed ($v_p$) and fiber speed ($v_f$) used for fiber drawing with the corresponding theoretical inner ($TD_{fi}$) and outer ($TD_{fo}$) diameters of the fibers.

| Fiber code | $V_p$ (mm/min) | $V_f$ (m/min) | $TD_{fi}$ (μm) | $TD_{fo}$ (μm) |
|---|---|---|---|---|
| FA | 0.3 | 2.5 | 71 | 124 |
| FB | 0.5 | 2.5 | 92 | 160 |

The fibers were observed using an optical microscope and the outer and inner diameters ($D_{fo}$ and $D_{fi}$) of the obtained fibers were measured on 20 fiber sections by image processing (ImageJ software).

### 2.2. Biological test with the fiber dissolution products

The effect of the fiber dissolution products was studied on a neonatal olfactory bulb ensheathing cell line (NOBEC), derived from primary cells dissociated from neonatal rat olfactory bulb and immortalized by retroviral transduction of SV40 large T antigen [22].

Two samples of hollow fibers (FB, 2 cm) were sterilized in an oven (180 °C, 3 h), soaked in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 100 units $ml^{-1}$ penicillin, 0.1 mg $ml^{-1}$ streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum (FBS; all from Invitrogen) at a (solution volume)/(fiber exposed surface) ratio of 1 ml/$cm^2$ and stored at 37 °C in a humidified atmosphere of 5% $CO_2$/air. Moreover, it was verified that the heat sterilization process (180 °C, 3 h) does not interfere with the possibility of filling the hollow fibers.

At different time points (3, 14, 21 and 28 days) the medium was collected and the fibers were washed in distilled water, dried in an oven at 37 °C and weighed. The weight loss percentage at the time point $i$ was calculated using the following formula:

$$\%WL_i = 100\,(W_0 - W_i)/W_0 \quad (3)$$

where $W_0$ and $W_i$ are, respectively, the sample weight at the beginning of the test and at the time point $i$.

The weight loss rate per unit area (WLR) was calculated dividing the final weight loss (WL) of the samples at the end of the test by the test duration $t$ and by the exposed surface area $A$, which was approximated as the inner and outer lateral surfaces of a hollow cylinder of length $L$ and inner and outer diameters equal to $D_{fi}$ and $D_{fo}$:

$$WLR = WL/(tA) = WL/\left[t\pi L\left(D_{fo} + D_{fi}\right)\right]. \quad (4)$$

In order to evaluate the dissolution product effects on cells, an experiment on cell line was carried out with media collected after 14 and 28 days of fiber dissolution (sample codes, respectively, 14F and 28F). As control media, samples of culture medium without fibers were maintained in the same conditions of the fiber-containing samples and then collected after 14 and 28 days ("aged" media, ctrl14, ctrl28). For each test also "fresh" culture medium (ctrl) was used. The media (14F, 28F, ctrl14, ctrl28) were supplemented with 100 units $ml^{-1}$ penicillin, 0.1 mg $ml^{-1}$ streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine and 10% heat-inactivated fetal bovine serum (FBS; all from Invitrogen) before in vitro tests.

About 2,000 cells/$cm^2$ were cultured in a 12-well plate in the presence of 1 ml of the media containing the fiber dissolution products (14F) and the control media (ctrl, ctrl14). After 1, 3 and 5 days of incubation, the cells were washed with phosphate buffered saline (PBS; Invitrogen) and detached by incubation with 500 μl of trypsin (Sigma-Aldrich) for 5–10 min at 37 °C. Complete medium was added to inactivate trypsin and then cells were counted using a Bürker's chamber. The study was carried out in triplicate and reported as mean value ± standard error of the mean.

About 27,000 cells/$cm^2$ were cultured in a 12-well plate in the presence of 1 ml of the medium containing the fiber dissolution products (28F) and the control media (ctrl, ctrl28). After 3 days of culture total proteins were extracted from 3 different wells by solubilizing cells in boiling Laemmli buffer (2.5% SDS and 0.125 M Tris–HCl pH 6.8), followed by 3 min at 100 °C. Protein concentration was determined by the bicinchoninic acid assay (BCA) method, and equal amounts of proteins (denatured at 100 °C in 240 mM 2-mercapto ethanol and 18% glycerol) were separated on a 12% polyacrylamide gel, consisting of acrylamide–bisacrylamide 12%, 0.375 M Tris pH 8.8, 0.1% SDS, 0.1%