Video Article

Designing Porous Silicon Films as Carriers of Nerve Growth Factor

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Abstract

Despite the great potential of NGF for treating neurodegenerative diseases, its therapeutic administration represents a significant challenge as the protein does not cross the blood-brain barrier, owing to its chemical properties, and thus requires long-term delivery to the brain to have a biological effect. This work describes fabrication of nanostructured PSi films as degradable carriers of NGF for sustained delivery of this sensitive protein. The PSi carriers are specifically tailored to obtain high loading efficacy and continuous release of NGF for a period of four weeks, while preserving its biological activity. The behavior of the NGF-PSi carriers as a NGF delivery system is investigated *in vitro* by examining their capability to induce neuronal differentiation and outgrowth of PC12 cells and dissociated DRG neurons. Cell viability in the presence of neat and NGF-loaded PSi carriers is evaluated. The bioactivity of NGF released from the PSi carriers is compared to the conventional treatment of repetitive free NGF administrations. PC12 cell differentiation is analyzed and characterized by the measurement of three different morphological parameters of differentiated cells; (i) the number of neurites extracting from the soma (ii) the total neurites' length and (iii) the number of branching points. PC12 cells treated with the NGF-PSi carriers demonstrate a profound differentiation throughout the release period. Furthermore, DRG neuronal cells cultured with the NGF-PSi carriers show an extensive neurite initiation, similar to neurons treated with repetitive free NGF administrations. The studied tunable carriers demonstrate the long-term implants for NGF release with a therapeutic potential for neurodegenerative diseases.

Video Link

The video component of this article can be found at https://www.jove.com/video/58982/

Introduction

NGF is essential for the development and maintenance of neurons in the peripheral nervous system (PNS)[1] and plays a crucial role in the survival and function of basal forebrain cholinergic neurons in the central nervous system (CNS)[2]. Its high pharmacological potential for treating central neurodegenerative diseases, such as Alzheimer's and Parkinson's, has been widely demonstrated, with clinical trials currently in progress[3,4,5,6]. The greatest challenge in the delivery of NGF to the CNS resides in its inability to cross the blood brain barrier (BBB), when systemically administered[7]. Moreover, NGF susceptibility to rapid enzymatic degradation renders its short half-life and significantly limits its therapeutic use[8,9]. Therefore, there is an unmet challenge to design delivery systems which allow for a prolonged and controlled release of NGF in a safe manner. Various NGF delivery systems, including polymer-based systems, have been studied[10,11,12,13,14,15,16,17]. The release profiles of these systems were often characterized by a distinct initial burst followed by a slow continuous release, where in the latter stage the release rate was significantly low in comparison to the initial burst[11,18,19]. Furthermore, inactivation of the protein by the acidic degradation products of the polymers (*e.g.*, poly(lactic-co-glycolic) acid) or loss of NGF bioactivity during the encapsulation process were observed with this systems[20].

Nanostructured PSi is characterized by several appealing properties, including its high surface area, large porous volume, biocompatibility, and tunable degradability in bodily fluids, predestining it for a promising drug delivery platform[21,22,23,24,25,26,27,28]. Proper selection of its anodization conditions allows to easily adjust the PSi structural properties (*e.g.*, porosity and pore size) for tailoring drug loading and release kinetics[21,27]. Moreover, various convenient chemical routes allow to modify the surface of the PSi and by that further tune the dissolution rate of the Si scaffold under physiological conditions and the release rates of the drug[22,24,29,30].

This work focuses on designing a PSi-based delivery system for prolonged controlled release of NGF. The effect of the NGF-PSi carriers on neuronal differentiation and outgrowth is examined using PC12 cells and dissociated DRG neurons. We demonstrate that the loaded NGF has retained its bioactivity by inducing neurite outgrowth and profound differentiation throughout a 1-month release period within a single administration.

Protocol

All methods have been approved by the Ethics Committee of Bar Ilan University.

1. Fabrication of Oxidized PSi ($PSiO_2$) Carriers

1. Cut a Si wafer (single side polished on the <100> face and heavily Boron-doped, p-type, 0.95 mΩ·cm) into 1.5 cm × 1.5 cm samples using a diamond-tipped pen.
2. Oxidize the Si samples in a tube furnace at 400 °C for 2 h in ambient air (heating rate: 25 °C/min, natural cooling).
3. Immerse the Si samples in a solution of aqueous hydrofluoric acid (HF) (48%), ddH_2O and ethanol (99.9%) (1:1:3 v/v/v) for 5 min; then rinse the samples with ethanol three times and dry under a nitrogen stream.
   NOTE: Prepare and store HF solution in plasticware only, as HF dissolves glass.
   CAUTION: HF is a highly corrosive liquid, and it should be handled with extreme care. In case of exposure, rinse thoroughly with water and treat the affected area with HF antidote gel; seek for medical care immediately.
4. Mount the Si sample in a polytetrafluoroethylene etching cell, using a strip of aluminum foil as a back-contact and a platinum coil as the counter electrode.
5. Etch a sacrificial layer in a 3:1 (v/v) solution of aqueous HF and ethanol (99.9%) for 30 s at a constant current density of 250 mA/cm^2; then rinse the surface of the resulting PSi film with ethanol three times and dry under a nitrogen stream.
6. Dissolve the freshly-etched porous layer in an aqueous NaOH solution (0.1 M) for 2 min. Then rinse with ethanol three times and dry under a nitrogen stream.
7. Immerse the sample in a solution of aqueous HF (48%), ddH_2O and ethanol (99.9%) (1:1:3 v/v) for 2 min. Then rinse with ethanol three times and dry under a nitrogen stream.
8. Electrochemically etch the Si sample in a 3:1 (v/v) solution of aqueous HF and ethanol (99.9%) for 20 s at a constant current density of 250 mA/cm^2; then rinse the surface of the resulting PSi film with ethanol three times and dry under a nitrogen stream.
9. Thermally oxidize the freshly-etched PSi samples in a tube furnace at 800 °C for 1 h in ambient air (heating rate: 25 °C/min, natural cooling) to form a porous SiO_2 ($PSiO_2$) scaffold.
10. Spin-coat the $PSiO_2$ samples with a positive thick photoresist at 4,000 rpm for 1 min; then bake the coated samples at 90 °C for 2 min (heating rate: 5 °C/min, natural cooling).
11. Dice the $PSiO_2$ samples into 8 mm × 8 mm samples using a dicing saw.
12. To remove the photoresist, soak the diced samples in acetone for 3 h; then thoroughly rinse with ethanol and dry under a nitrogen stream.

2. Loading $PSiO_2$ with NGF

1. To prepare the NGF loading solution, dissolve 20 µg of murine β-NGF in 400 µL of 1:1 (v/v) solution of 0.01 M phosphate-buffered saline (PBS) and ddH_2O.
2. Add 52 µL of the loading solution on top of the $PSiO_2$ sample and incubate for 2 h at RT in a capped dish.
   NOTE: Maintain high humidity in the dish to prevent drying up of the solution during incubation.
3. Collect the solution on the top of the sample for subsequent quantification of NGF content within the $PSiO_2$ carrier.
   NOTE: NGF loading into $PSiO_2$ should be performed immediately before the intended use, the protocol cannot be paused here due to the risk of drying and denaturation of the protein.

3. Quantification of NGF loading by NGF ELISA

1. To prepare the ELISA plate, add 100 µL of 0.5 µg/mL capture antibody (rabbit anti-hβ-NGF) to each well, seal the plate and incubate overnight at RT.
2. Aspirate the wells to remove liquid and wash the plate four times using 300 µL of wash buffer (0.05% Tween-20 in PBS) per well; after the last wash invert the plate to remove residual buffer and blot on a paper towel.
3. Add 300 µL of block buffer (1% bovine serum albumin (BSA) in PBS) to each well and incubate for at least 1 h at RT. Then aspirate and wash the plate four times as described in step 3.2.
4. To prepare a calibration curve, dilute NGF loading solution (see step 2.1) in diluent (0.05% Tween-20, 0.1% BSA in PBS) to 1 ng/mL. Then perform 2-fold serial dilutions from 1 ng/mL to zero to obtain the calibration curve samples.
5. To prepare the samples, dilute the NGF loading solution (from step 2.1) and the collected post-loading solution (from step 2.3) in diluent 1:100,000 and 1:10,000 respectively, to reach the concentrations range of the ELISA kit in use (16-1,000 pg/mL).
6. Add 100 µL of the diluted samples and calibration curve samples to each well in triplicate and incubate at RT for 2 h; then aspirate and wash the plate four times as described in step 3.2.
7. Add 100 µL of 1 µg/mL detection antibody (biotinylated rabbit anti-hβ-NGF) to each well and incubate at RT for 2 h. Then aspirate and wash the plate four times as described in step 3.2.
8. Dilute Avidin-Horseradish Peroxidase (HRP) 1:2,000 in diluent, add 100 µL per well and incubate for 30 min at RT. Now aspirate and wash the plate four times as described in step 3.2.
9. Add 100 µL of substrate solution to each well and incubate 25 min at RT for color development. Measure the absorbance at 405 nm with wavelength correction set at 650 nm using a microplate reader.
10. Determine NGF concentration in both the loading solution and the collected post-loading solution based on the calibration curve.
11. To calculate NGF mass loaded within $PSiO_2$ carrier, subtract NGF mass in the collected post-loading solution from the NGF mass in loading solution. NGF loading efficacy is calculated by the following equation:

$$\text{NGF loading efficacy [\%]} = \frac{\text{Weight of NGF in PSiO}_2\text{ carrier}}{\text{Weight of NGF in loading solution}} \times 100$$

4. *In vitro* NGF Release from $PSiO_2$

1. Incubate the NGF-loaded $PSiO_2$ carriers in 2 mL of 0.01 M PBS containing 1% (w/v) BSA and 0.02% (w/v) sodium azide at 37 °C and under orbital agitation of 100 rpm.
2. Every 2 days collect the solution and replace it with 2 mL of fresh PBS. Freeze the collected release samples in liquid nitrogen and store at -20 °C for further analyses.
3. Use the commercially available NGF ELISA assay to quantify NGF content in the release samples as described in steps 3.1-3.9.
   NOTE: When preparing the release samples for ELISA quantification, different dilutions should be performed for different time points along the release period (*i.e.,* earlier time points should be diluted much more than later time points). Moreover, for each release sample, at least two different dilutions should be performed and quantified to ensure reaching the concentrations range of the ELISA kit.
4. Determine NGF concentration in the release samples based on the calibration curve and plot a graph of accumulative NGF release over time.

5. Quantification of *in vitro* Si Erosion by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES)

1. Dilute 1 mL of release samples 1:10 in release buffer (0.01 M PBS containing 1% (w/v) BSA and 0.02% (w/v) sodium azide) for a total volume of 10 mL.
2. Monitor Si atomic emission peaks at 212.4, 251.6 and 288.2 nm for the diluted samples.
3. Determine Si concentration in the samples based on a calibration curve.
4. To establish the Si degradation profile, express Si content at each time point as a percentage of the total Si content of the carriers (*i.e.,* the cumulative Si content along the release period).
   NOTE: To ensure accurate quantification of the total Si content of the carriers, release samples are sampled 1 month following the end-point of the release study and analyzed for Si concentration using ICP-AES. Summing the cumulative Si content along the release period and the Si content in the post-release samples provides the 100% of Si content.

6. Cell Viability and Growth in the Presence of NGF-Loaded $Psio_2$ Carriers

1. **Rat pheochromocytoma (PC12) cell culture**
   1. Prepare the basic growth medium by adding 10% horse serum (HS), 5% fetal bovine serum (FBS), 1% L-glutamine, 1% penicillin streptomycin and 0.2% amphotericin to Roselle Park Medical Institute (RPMI) medium.
   2. Prepare differentiation medium by adding 1% HS, 1% L-glutamine, 1% penicillin streptomycin and 0.2% amphotericin to RPMI medium.
   3. Grow cell suspension (10^6 cells) in a 75 cm^2 culture flask with 10 mL of basic growth medium for 8 days; every 2 days add 10 mL of basic growth medium to the flask.
   4. To generate differentiated PC12 cell culture, transfer the cell suspension to a centrifuge tube; centrifuge cells for 8 min at 200 x *g* and RT. Discard the supernatant.
   5. Suspend the cells in 5 mL of fresh basic growth medium and re-centrifuge the cells for 5 min at 200 x *g* and RT; discard the supernatant and resuspend the cell pellet in 3 mL of basic growth medium.
   6. To separate cell clusters, aspirate the cells ten times using a 23 G syringe.
   7. Count the cells using a hemocytometer cell counter and seed 10^4 cells/cm^2 working area on collagen type I coated plates in the presence of differentiation medium.
   8. After 24 h, add fresh murine β-NGF (50 ng/mL) or NGF-loaded PSiO2 carrier per plate.
      NOTE: Higher NGF concentrations (>50 ng/mL) possess the exact effect as the specified concentration.
   9. Renew the differentiation medium every 2 days.
   10. To evaluate cell viability, add 10% (v/v) of the viability indicator solution (resazurin-based) at representative time points and incubate for 5 h at 37 °C; measure the absorbance at 490 nm using a spectrophotometer.

2. **Mice Dorsal Root Ganglia (DRG) Cell Culture**
   1. To isolate DRGs from two 7-week-old C57bl mice, first douse the mice with 70% ethanol and make an incision to remove the skin.
   2. Cut the base of the skull and the abdominal wall muscles till the spinal cord is exposed.
   3. Remove the spinal column by making a cut at the level of the femurs and clean the surrounding tissues.
   4. Cut the column into three pieces; then cut each piece in the midline to generate two halves and peel out the spinal cord.
   5. Under the binocular, extract all DRG ganglia and immerse them in a cold Hank's balanced salt solution (HBSS).
   6. Clean the surrounding connective tissues by pining the DRGs on a petri dish and gently remove residual meninges under the binocular.
   7. Dissociate the DRG cells by incubating them for 20 min in 1,000 U of papain.
   8. Centrifuge the cells for 4 min at 400 x *g*. Discard the supernatant and keep the cell pellet.
   9. Resuspend the pellet in a 10 mg/mL collagenase and 12 mg/mL dispase-II solution and incubate for 20 min at 37 °C.
   10. Centrifuge the cells for 2 min at 400 x *g*; discard the supernatant and keep the cell pellet.
   11. Triturate the cell pellet in 1 mL of HBSS, 10 mM glucose and 5 mM HEPES (pH 7.35) by repeated passage through a constricted Pasteur pipette.
   12. Gently add the dissociated cells to L-15 medium containing 20% of silica-based colloidal medium for cell separation by density gradient centrifugation; centrifuge for 8 min at 1,000 x *g*. Aspirate the supernatant and keep the cell pellet.
       NOTE: The aim of this step is to separate and purify the neuronal cells from axonal debris, Schwann cells and fibroblasts.
   13. Resuspend the cell pellet in 2 mL of F-12 medium; centrifuge for 2 min at 1,000 x g; discard the supernatant and resuspend the pellet in 1 mL of F-12 medium.

14. Count the cells and seed 2 × 10^4 cells on poly-L-lysine and laminin coated glass bottom 35 mm culture dishes, in a F-12 antibiotic-free medium supplemented with 10% FBS.
    NOTE: Initially, seed the cells in a small volume of medium (150-200 µL); following 2 h incubation at 37 °C, add medium for a final volume of 2 mL.
15. Gently add the NGF-loaded $PSiO_2$ carrier or fresh murine β-NGF (50 ng/mL) per plate.
16. After 2 days, fix the cell culture by incubating it with a 4% paraformaldehyde (PFA) solution for 15 min at RT; immunostain the cell culture using a common immunofluorescence staining procedure[31].
17. Image the neuronal cell culture using a confocal microscope.

7. Cell Differentiation Analysis

1. **PC12 cells differentiation percentage in the presence of NGF-loaded $PSiO_2$ carriers**
    1. Incubate the NGF-loaded $PSiO_2$ carriers in 2 mL of differentiation medium, in a humidified incubator at 37 °C containing 5% CO_2.
    2. Every 2 days, collect the solution and replace it with 2 mL of fresh medium. Freeze the collected release samples in liquid nitrogen and store at -20 °C for further analyses.
    3. Seed PC12 cells in 12-well collagen-coated plates as instructed in section 4.1 (4.1.3-4.1.6).
    4. After 24 h, introduce the release samples of representative time points (from section 5.1.2) to the different wells; for control wells, add fresh murine β-NGF (50 ng/mL).
    5. After 24 h, image the cells using a light microscope.
    6. To determine the percentage of neurite-bearing cells, manually count cells that had outgrown neurites out of the total number of cells in the images (n = 5 frames for each well); plot a graph of percentage of PC12 cells with neurite outgrowth over time.
       NOTE: Undifferentiated PC12 cells appeared to have rounded structure without neurites, while differentiated cells can be identified by outgrowth of neurites (at least one of minimum length equal to the cell soma diameter) or morphological changes.

2. **Morphometric analysis of differentiated PC12 cells**
    1. For image processing analysis, acquire phase images of cultured cells up to 3 days after treatment with NGF (as free reagent or as the release product of the NGF-loaded $PSiO_2$ carriers).
       NOTE: At later time points (beyond day 5) the cells develop highly complex networks, preventing morphometric measurements at a single cell resolution.
    2. Download the image processing program NeuronJ, an ImageJ plug-in, which enables a semi-automatic neurite tracing and length measuring.
    3. Convert the image file to an 8-bit format and measure pixel to micrometer ratio using the image scale bar.
    4. Set the scale using the **Analyze** | **Set Scale** command and measure the length of each neurite.
    5. Manually count the number of branching points and the number of neurites originating from each cell soma.
       NOTE: An average neurite length 1 day after NGF treatment is approximately 30 µm. The measured neurite lengths may be widely distributed.

Representative Results

Oxidized PSi films are fabricated as described in the protocol. The Si wafer is subjected to electrochemical etching for 20 s at 250 mA/cm^2 (**Figure 1ai**), followed by thermal oxidation at 800 °C (**Figure 1aii**) to produce a $PSiO_2$ scaffold. High-resolution scanning electron microscopy (HR-SEM) images of the resulting $PSiO_2$ film are shown in **Figure 1b, c.** Top-view micrograph of the film (**Figure 1b**) depicts its highly porous nature with pores of approximately 40 nm in diameter. Cross-sectional micrograph of a cleaved film (**Figure 1c**) reveals a porous layer thickness of 2.9 µm, characterized by interconnecting cylindrical pores.

The $PSiO_2$ films are loaded with the NGF loading solution as illustrated in **Figure 1aiii**. NGF loading is quantified using NGF ELISA by measuring the NGF concentrations in the loading solution before and after incubation with the $PSiO_2$ films. The average mass of the loaded NGF per $PSiO_2$ film is determined as 2.8 ± 0.2 µg, which corresponds to a high loading efficacy of 90% (w/w) (data not shown[31]). In order to establish the NGF release profile from the $PSiO_2$ carriers, the loaded films are incubated in PBS at 37 °C and every 2 days aliquots are sampled for quantification of the released protein concentration using NGF ELISA. In addition, the Si content in the release samples is quantified using ICP-AES and the Si erosion kinetics of the $PSiO_2$ carriers is established. **Figure 2** depicts the NGF release and the corresponding Si degradation profiles of the porous carriers. A sustained release of NGF, without burst effect, is attained for a period of 1 month. NGF release in the first week is faster (slope = 0.442) compared to a much slower release in later days along the release period (slope 0.043). It should be noted that throughout the entire 1-month release period, the amount of NGF released is sufficient for inducing profound differentiation of PC12 cells, as will be discussed later. The accumulative NGF released is found to be in a good correlation (R^2 = 0.971) with the remaining Si content, as shown in the inset of **Figure 2**.

Next, the bioactivity of the NGF-loaded $PSiO_2$ carriers is characterized *in vitro*, PC12 cells and DRG neurons are used as models for neuronal differentiation and outgrowth. PC12 cells and dissociated DRG neurons are seeded as described in the protocol. First, cell viability in the presence of the $PSiO_2$ carriers is examined by incubating the cells with neat (not loaded) or NGF-loaded $PSiO_2$; control plates and cells treated with neat $PSiO_2$ are supplemented with free NGF every 2 days. No cytotoxicity is observed upon exposure of the cells to the neat or NGF-loaded $PSiO_2$ carriers (**Figure 3a**), demonstrating that $PSiO_2$ is biocompatible with this cell line. **Figure 3b** shows representative HR-SEM micrographs of PC12 cells treated with the NGF-loaded $PSiO_2$ carriers. The observed cells extract neurites and form characteristic branched neuronal networks. Similar results are obtained when seeding dissociated DRG neuronal cells in the presence of the NGF-loaded $PSiO_2$ carriers. Confocal images of immunostained DRG cells cultured with the NGF-loaded $PSiO_2$ carriers (**Figure 3e**) show an extensive neurite initiation and elongation, similar to the positive control (*i.e.*, neurons supplemented with free NGF, see **Figure 3d**), while the negative control (*i.e.*, untreated neurons, see **Figure 3c**), exhibits a poor extent of neurite outgrowth.

To evaluate PC12 cells differentiation percentage in the presence of NGF released from the $PSiO_2$ carriers, differentiation is quantified by counting the cells with neurite outgrowth out of the total cell population. **Figure 4** summarizes the results in terms of the percentage of differentiated cells at different time points over a 26-day period. Significant differentiation percentage values are attained throughout the studied duration. Notably, after 26 days, the released NGF has induced differentiation of above 50%, a differentiation percentage which is significantly higher compared to previous studies with polymer-based carriers[16]. These results indicate that NGF entrapment within the porous host preserved the biological activity of the protein for inducing profound differentiation for a period of ~1 month.

To further examine the effect of NGF-$PSiO_2$ carriers on neuronal differentiation, three characteristic morphological parameters of the differentiated PC12 cells are measured at the single cell level: (i) the number of neurites extracting from the soma (ii) the total neurites' length and (iii) the number of branching points. The cells are treated with NGF-loaded $PSiO_2$ carriers or with repetitive administration of free NGF (every 2 days), as a control. **Figure 5a-c** presents the morphometric analysis of the cell population at days 1 and 3. The PC12 cells treated with the NGF-loaded $PSiO_2$ show morphometric values which are similar to the control treatment for all three tested parameters. After 3 days, the values of all morphological parameters are observed to increase at the same rate for both the NGF-loaded $PSiO_2$ carriers and the control treatment of repetitive administration of free NGF.

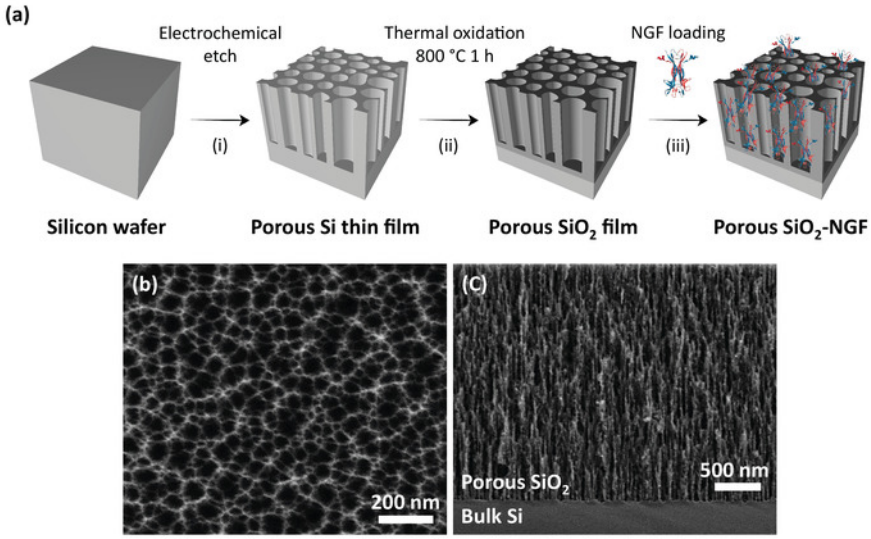

Figure 1: Fabrication of $PSiO_2$ films. (a) Fabrication scheme of the $PSiO_2$ carriers: (i) Silicon wafer is subjected to electrochemical etching for 20 s at 250 mA/cm^2, followed by (ii) thermal oxidation at 800 °C to produce a $PSiO_2$ scaffold, and (iii) NGF loading by physical adsorption (schematics are not drawn to scale).**(b-c)** Top-view and cross-section electron micrographs of a characteristic $PSiO_2$ film. Please click here to view a larger version of this figure.

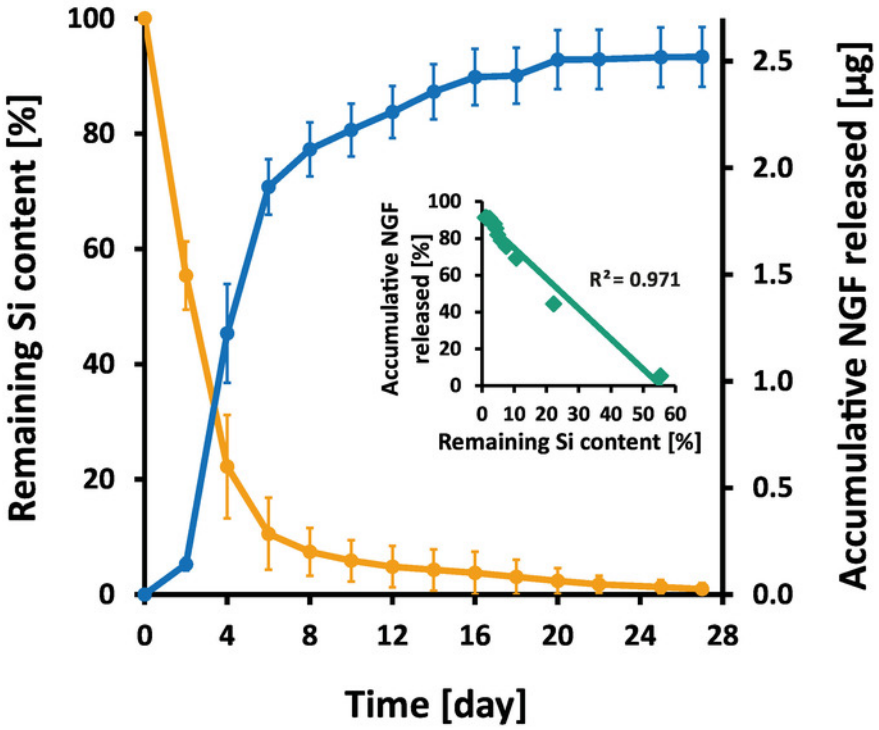

Figure 2: NGF release and Si erosion profiles of NGF-loaded $PSiO_2$ carriers. The extent of degradation of the $PSiO_2$ carriers is presented as the fraction of the remaining Si content at each time point out of the total Si content of the porous film. The inset shows the correlation between the accumulative NGF released and remaining Si content. Error bars represent SD, n=3. Please click here to view a larger version of this figure.

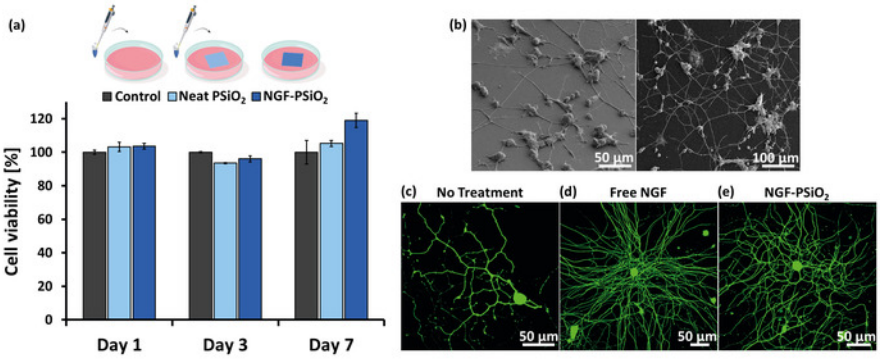

Figure 3: Cell viability and growth in the presence of NGF-loaded $PSiO_2$ carriers. (a) *In vitro* cytotoxicity characterization. The PC12 cells viability is measured on 1, 3 and 7 days following their exposure to neat (not loaded) $PSiO_2$ carriers, NGF-loaded $PSiO_2$ carriers or free NGF (50 ng/mL every 2 days, control plates). **(b)** Electron micrographs of PC12 cells cultured with NGF-loaded $PSiO_2$ carriers for 9 days. Left panel: a zoom-in, depicting neurite outgrowth from the cell soma; right panel: a zoom-out, indicating the highly complex neuronal network formed. **(c-e)** Confocal microscopy images of immunostained DRG neuronal cell culture (2 days after seeding): (**c**) untreated cells; (**d**) cells supplemented with free NGF (50 ng/mL); (**e**) NGF-$PSiO_2$ carriers. Immunofluorescent staining of neurofilament H enables imaging of the cell soma and the outgrowing neurites. Scale bar = 50 µm. Please click here to view a larger version of this figure.

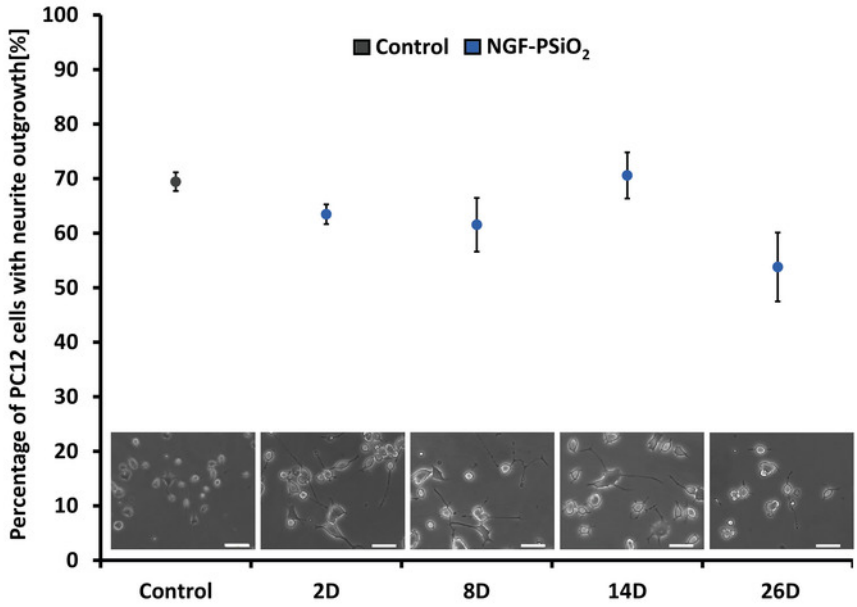

Figure 4: PC12 cells differentiation percentage values upon exposure to NGF released from NGF-loaded $PSiO_2$ carriers at representative time points. Differentiation percentage is expressed as the number of cells with neurite outgrowth out of the total cell population. Control treatment refers to cells supplemented with free NGF (50 ng/mL). Representative light microscopy images of the cells at the different time points are depicted along the x-axis; scale bar = 25 µm for micrographs of days 2, 8, 14, 26 and 50 µm for control micrograph. Error bars represent SD, n=3. Please click here to view a larger version of this figure.

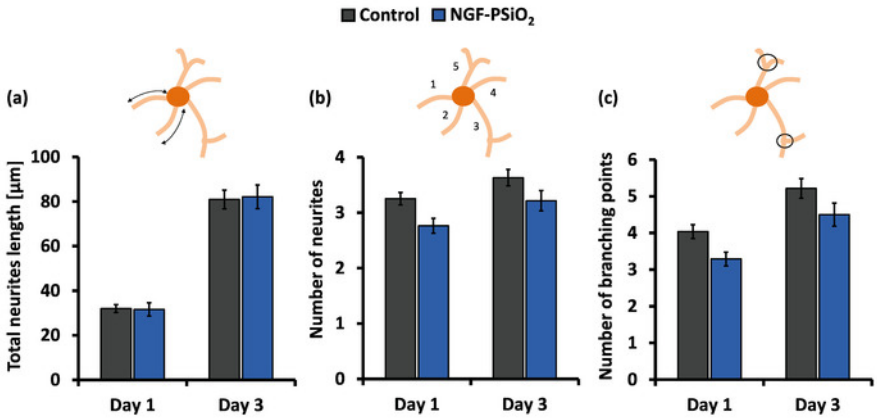

Figure 5: Morphometric analysis of differentiated PC12 cells. Three morphological parameters of differentiated PC12 cells are measured, at the single cell level, at day 1 and 3 following exposure to NGF-loaded $PSiO_2$ carriers or repetitive administration of free NGF every 2 days (control); (**a**) total neurites' length (**b**) number of neurites extracting from the cell soma and (**c**) number of branching points. Error bars represent SD, n=3. Please click here to view a larger version of this figure.

Discussion

Degradable nanostructured $PSiO_2$ films are fabricated and employed as carriers for NGF, allowing for its continuous and prolonged release, whilst retaining its biological activity. The potential of the $PSiO_2$ to serve as a delivery system for NGF is demonstrated *in vitro* by demonstrating their ability to release sufficient NGF dosage to induce neuronal differentiation and promote outgrowth of PC12 cells and DRG neurons. The engineered films can be used as long-term reservoirs of NGF for future treatment *in vivo*.

The structural properties of the fabricated PSi films were tailored specifically for NGF payload; the current density of the electrochemical etching process was adjusted to obtain pore size of approximately 40 nm that would easily accommodate the NGF, a protein with a molecular weight of 26.5 kDa[32] and a characteristic diameter of ~4 nm[33], within the porous matrix. Moreover, thermal oxidation of the porous scaffold was performed to enable physical adsorption of NGF by electrostatic attraction of the positively charged protein to the negatively charged oxidized PSi surface. The surface chemistry of PSi exerts a major effect on the loading efficacy and can be easily tuned in order to better control the interactions between the payload and the porous matrix. These interactions subsequently dictate the structure of adsorbed protein molecules and their bioactivity[34,35,36]. In conclusion, the system was adjusted to obtain optimal loading of NGF by carefully selecting the appropriate pore size, surface characteristics and the ideal loading solvent and the resulting effect of the mentioned parameters dictates the protein loading efficacy.

Therefore, any change in the fabrication parameters (*e.g.*, current density, etching time, type and concentration of dopant or electrolyte), surface chemistry or loading solution composition can affect the loading efficacy and bioactivity of the loaded protein.

The release rate of a payload from the PSi or$PSiO_2$host is generally dictated by a combination of two simultaneous mechanisms, out-diffusion of the payload molecules and the degradation of the Si scaffold[37]. The erosion and subsequent dissolution rate are affected by the implantation site, its pathology and disease state[28,38,39]. It was established in previous work that if a different release rate is required for a certain therapeutic application, the release profile can be modified and prolonged by changing the surface chemistry of the PSi surface[38,40,41]. Various chemical modifications, such as thermal oxidation, thermal carbonization and hydrosilylation techniques, have been shown to stabilize the PSi surface and affect its degradation and consequent payload release[35,42,43,44,45]. Moreover, loading of NGF into the carriers by covalent attachment of the protein molecules to the Si scaffold via various surface chemistry routes should result in a more prolonged release because the payload is only released when the covalent bonds are broken or the supporting Si matrix is degraded[21].

Furthermore, following its fabrication process, PSi can be rendered into various configurations besides thin films, such as microparticles[46], nanoparticles[47] or free-standing membranes[26], which can also be employed as carriers for NGF and meet specific application needs.

In order to be clinically relevant, the NGF content within the $PSiO_2$ carriers should reach the range of therapeutic doses. In the method described in the protocol, the NGF-loaded $PSiO_2$ carriers are introduced into a consistent volume of 2 mL of cell media or PBS buffer and thus, the concentration of the loading solution and the respective NGF mass loaded were adjusted to yield a released NGF concentration that is relevant for the tested *in vitro* system. When utilizing this method for different systems, such as *ex vivo* or *in vivo* environments, the concentration of the NGF loading solution should be increased and adjusted according to the needed dose. Alternatively, higher NGF content can be obtained by introducing multiple carriers per tested area or by using larger areas of $PSiO_2$ samples.

Moreover, it should be noted that in later time points along the release period, the released NGF concentrations are much lower than in earlier time points. The fact that the NGF flux is not constant over time must be taken into consideration when designing the system according to application needs.

Numerous NGF delivery systems have been developed and reported in the literature, most of them are polymer-based systems, comprising of synthetic or natural polymer conjugates[10,11,12,15,16,17]. These systems have shown effective sustained release profiles, however, the release period spanned over a period of several days with a significant burst effect. Some of these delivery platforms suffer from critical limitations such as loss of bioactivity upon the encapsulation process, requiring usage of different stabilizing agents[18,48], as well as sophisticated and complex fabrication techniques[16]. One of the greatest challenges in designing delivery systems for proteins is the ability to preserve the bioactivity of the molecules upon entrapment within the carrier system. Proteins or peptides can be loaded into PSi/$PSiO_2$ at RT or even at lower temperatures without using strong organic solvents, which are both important factors when loading these sensitive biomolecules. Previous studies have demonstrated that PSi/$PSiO_2$ surface chemistry plays a crucial role in minimizing possible denaturation of the loaded proteins[35,36]. Therefore, PSi/$PSiO_2$ is an advantageous nanomaterial for developing delivery systems for growth factors in general and NGF in particular.

The current work is focused on utilizing this method as a new therapeutic approach for direct administration of NGF into the CNS for potential treatment of neurodegenerative diseases. The NGF-loaded $PSiO_2$ carriers can be implanted in mice brains and the efficacy of the platform as long-term implants is studied *in vivo*. Furthermore, combining this promising carriers with non-invasive biolistics[49,50] may enable one to administer the NGF-loaded $PSiO_2$ particles in a highly spatial resolution to a localized area using a novel pneumatic capillary gun for treating neurodegenerative disorders, where a spatiotemporal drug administration is required. Moreover, NGF can direct neuronal growth in a chemical gradient manner[51], similar to axon guidance molecules. Thus, the loaded $PSiO_2$ carriers can serve as attractant hot spots to NGF, to direct growth, complementary to other directing cues[52,53]. In addition, the $PSiO_2$carriers can be specifically tailored to sustain the delivery of NGF for a much-extended time period of up to several months by further tuning the $PSiO_2$ nanostructure and its surface chemistry.

Disclosures

The authors declare no competing financial interests.

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