Journal of Visualized Experiments

Designing Porous Silicon Films as Carriers of Nerve Growth Factor

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58982R2
Full Title:	Designing Porous Silicon Films as Carriers of Nerve Growth Factor
Keywords:	Designing Porous Silicon Films as Carriers of Nerve Growth Factor for Promoting Differentiation and Outgrowth of PC12 Cells and Dorsal Root Ganglion
Corresponding Author:	Ester Segal Technion Israel Institute of Technology Haifa, Israel ISRAEL
Corresponding Author's Institution:	Technion Israel Institute of Technology
Corresponding Author E-Mail:	esegal@technion.ac.il
Order of Authors:	Michal Rosenberg
	Neta Zilony
	Orit Shefi
	Ester Segal
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$4,200)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Haifa and Ramat Gan, Israel

TITLE:

Designing Porous Silicon Films as Carriers of Nerve Growth Factor

AUTHORS AND AFFILIATIONS:

Michal Rosenberg[1], Neta Zilony[2,3,*], Orit Shefi[2,3] and Ester Segal[1,4]

[1]Department of Biotechnology and Food Engineering, Technion – Israel Institute of Technology, Technion City, Haifa, Israel

[2]Faculty of Engineering, Bar-Ilan University, Ramat-Gan, Israel

[3]Bar-Ilan Institute of Nanotechnologies and Advanced Materials, Ramat-Gan, Israel

[4]Russell Berrie Nanotechnology Institute, Technion – Israel Institute of Technology, Technion City, Haifa, Israel

[*]The authors contributed equally to this work

Corresponding Authors:

Ester Segal (esegal@technion.ac.il)

Orit Shefi (orit.shefi@biu.ac.il)

Email Addresses of Co-authors:

Neta Zilony (neta.zilony@biu.ac.il)

Michal Rosenberg (rosenbem@campus.technion.ac.il)

KEYWORDS:

Nerve Growth Factor; Porous Silicon; PC12; Dorsal Root Ganglia; Controlled Release; Neuronal Differentiation.

SUMMARY:

Here, we present a protocol to design and fabricate nanostructured porous silicon (PSi) films as degradable carriers for the nerve growth factor (NGF). Neuronal differentiation and outgrowth of PC12 cells and mice dorsal root ganglion (DRG) neurons are characterized upon treatment with the NGF-loaded PSi carriers.

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.

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[3-6]. 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[10-17]. 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[21-28]. 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

1.1. 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.

1.2. Oxidize the Si samples in a tube furnace at 400 °C for 2 h in ambient air (heating rate: 25 °C/min, natural cooling).

1.3. 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.

1.4. 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.

1.5. 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.

1.6. 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.

1.7. 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.

1.8. 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.

1.9. 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.

1.10. Spin-coat the $PSiO_2$ samples with a positive thick photoresist at 4000 rpm for 1 min; then bake the coated samples at 90 °C for 2 min (heating rate: 5 °C/min, natural cooling).

1.11. Dice the $PSiO_2$ samples into 8 mm × 8 mm samples using a dicing saw.

1.12. 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

2.1. 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.

2.2. 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.

2.3. 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

3.1. 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.

3.2. 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.

3.3. 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.

3.4. 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.

3.5. 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).

3.6. 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.

3.7. 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.

3.8. Dilute Avidin-Horseradish Peroxidase (HRP) 1:2000 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.

3.9. 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.

3.10. Determine NGF concentration in both the loading solution and the collected post-loading solution based on the calibration curve.

3.11. 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 $PSiO_2$

4.1. 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.

4.2. 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.

4.3. 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.

4.4. 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)

5.1. 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.

5.2. Monitor Si atomic emission peaks at 212.4, 251.6 and 288.2 nm for the diluted samples.

5.3. Determine Si concentration in the samples based on a calibration curve.

5.4. 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

6.1. Rat pheochromocytoma (PC12) cell culture

6.1.1. 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.

6.1.2. Prepare differentiation medium by adding 1% HS, 1% L-glutamine, 1% penicillin streptomycin and 0.2% amphotericin to RPMI medium.

6.1.3. 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.

6.1.4. 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.

6.1.5. 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.

6.1.6. To separate cell clusters, aspirate the cells ten times using a 23 G syringe.

6.1.7. 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.

6.1.8. 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.

6.1.9. Renew the differentiation medium every 2 days.

6.1.10. 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.

6.2. Mice Dorsal Root Ganglia (DRG) Cell Culture

6.2.1. 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.

6.2.2. Cut the base of the skull and the abdominal wall muscles till the spinal cord is exposed.

6.2.3. Remove the spinal column by making a cut at the level of the femurs and clean the surrounding tissues.

6.2.4. Cut the column into three pieces; then cut each piece in the midline to generate two halves and peel out the spinal cord.

6.2.5. Under the binocular, extract all DRG ganglia and immerse them in a cold Hank's balanced salt solution (HBSS).

6.2.6. Clean the surrounding connective tissues by pining the DRGs on a petri dish and gently remove residual meninges under the binocular.

6.2.7. Dissociate the DRG cells by incubating them for 20 min in 1000 U of papain.

6.2.8. Centrifuge the cells for 4 min at 400 x g. Discard the supernatant and keep the cell pellet.

6.2.9. Resuspend the pellet in a 10 mg/mL collagenase and 12 mg/mL dispase-II solution and incubate for 20 min at 37 °C.

6.2.10. Centrifuge the cells for 2 min at 400 x g; discard the supernatant and keep the cell pellet.

6.2.11. 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.

6.2.12. 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 1000 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.

6.2.13. Resuspend the cell pellet in 2 mL of F-12 medium; centrifuge for 2 min at 1000 x g; discard the supernatant and resuspend the pellet in 1 mL of F-12 medium.

6.2.14. 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.

6.2.15. Gently add the NGF-loaded $PSiO_2$ carrier or fresh murine β-NGF (50 ng/mL) per plate.

6.2.16. 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].

6.2.17. Image the neuronal cell culture using a confocal microscope.

7. Cell Differentiation Analysis

7.1. PC12 cells differentiation percentage in the presence of NGF-loaded $PSiO_2$ carriers

7.1.1. Incubate the NGF-loaded $PSiO_2$ carriers in 2 mL of differentiation medium, in a humidified incubator at 37 °C containing 5% CO_2.

7.1.2. 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.

7.1.3. Seed PC12 cells in 12-well collagen-coated plates as instructed in section 4.1 (4.1.3-4.1.6).

7.1.4. 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).

7.1.5. After 24 h, image the cells using a light microscope.

7.1.6. 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.

7.2. Morphometric analysis of differentiated PC12 cells

7.2.1. 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.

7.2.2. Download the image processing program NeuronJ, an ImageJ plug-in, which enables a semi-automatic neurite tracing and length measuring.

7.2.3. Convert the image file to an 8-bit format and measure pixel to micrometer ratio using the image scale bar.

7.2.4. Set the scale using the **Analyze** | **Set Scale** command and measure the length of each neurite.

7.2.5. 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 AND TABLE LEGENDS:

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.

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.

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.

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.

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.

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[34-36]. 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 requited 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[35,42-45]. 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[10-12,15-

[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.

ACKNOWLEDGMENTS:

MS and ES acknowledge the core services and support from the Lorry I. Lokey Center for Life Science and Engineering and the financial support of the Russell Berrie Nanotechnology Institute at the Technion.

DISCLOSURES:

The authors declare no competing financial interests.

REFERENCES

1 Wiesmann, C. & De Vos, A. Nerve growth factor: structure and function. *Cellular and Molecular Life Sciences.* **58** (5), 748-759 (2001).

2 Dreyfus, C. F. Effects of nerve growth factor on cholinergic brain neurons. *Trends in Pharmacological Sciences.* **10** (4), 145-149 (1989).

3 Eriksdotter Jönhagen, M. *et al.* Intracerebroventricular infusion of nerve growth factor in three patients with Alzheimer's disease. *Dementia and Geriatric Cognitive Disorders.* **9** (5), 246-257 (1998).

4 Eriksdotter-Jönhagen, M. *et al.* Encapsulated cell biodelivery of nerve growth factor to the basal forebrain in patients with Alzheimer's disease. *Dementia and Geriatric Cognitive*

Disorders. **33** (1), 18-28 (2012).
5 Eyjolfsdottir, H. *et al.* Targeted delivery of nerve growth factor to the cholinergic basal forebrain of Alzheimer's disease patients: application of a second-generation encapsulated cell biodelivery device. *Alzheimer's Research & Therapy.* **8** (1), 30 (2016).
6 Tuszynski, M. H. *et al.* A phase 1 clinical trial of nerve growth factor gene therapy for Alzheimer disease. *Nature Medicine.* **11** (5), 551-555 (2005).
7 Pan, W., Banks, W. A. & Kastin, A. J. Permeability of the blood–brain barrier to neurotrophins. *Brain Research.* **788** (1), 87-94 (1998).
8 Thorne, R. G. & Frey, W. H. Delivery of neurotrophic factors to the central nervous system. *Clinical Pharmacokinetics.* **40** (12), 907-946 (2001).
9 Tria, M. A., Fusco, M., Vantini, G. & Mariot, R. Pharmacokinetics of nerve growth factor (NGF) following different routes of administration to adult rats. *Experimental Neurology.* **127** (2), 178-183 (1994).
10 Bhang, S. H. *et al.* The effect of the controlled release of nerve growth factor from collagen gel on the efficiency of neural cell culture. *Biomaterials.* **30** (1), 126-132 (2009).
11 Camarata, P. J., Suryanarayanan, R., Turner, D. A., Parker, R. G. & Ebner, T. J. Sustained release of nerve growth factor from biodegradable polymer microspheres. *Neurosurgery.* **30** (3), 313-319 (1992).
12 Cooper, A., Bhattarai, N. & Zhang, M. Fabrication and cellular compatibility of aligned chitosan–PCL fibers for nerve tissue regeneration. *Carbohydrate Polymers.* **85** (1), 149-156 (2011).
13 Marcus, M. *et al.* Iron oxide nanoparticles for neuronal cell applications: uptake study and magnetic manipulations. *Journal of Nanobiotechnology.* **14** (1), 37 (2016).
14 Marcus, M., Skaat, H., Alon, N., Margel, S. & Shefi, O. NGF-conjugated iron oxide nanoparticles promote differentiation and outgrowth of PC12 cells. *Nanoscale.* **7** (3), 1058-1066 (2015).
15 Sridhar, R. *et al.* Electrosprayed nanoparticles and electrospun nanofibers based on natural materials: applications in tissue regeneration, drug delivery and pharmaceuticals. *Chemical Society Reviews.* **44** (3), 790-814 (2015).
16 Valmikinathan, C. M., Defroda, S. & Yu, X. Polycaprolactone and bovine serum albumin based nanofibers for controlled release of nerve growth factor. *Biomacromolecules.* **10** (5), 1084-1089 (2009).
17 Xu, X. *et al.* Polyphosphoester microspheres for sustained release of biologically active nerve growth factor. *Biomaterials.* **23** (17), 3765-3772 (2002).
18 Cao, X. & Shoichet, M. S. Delivering neuroactive molecules from biodegradable microspheres for application in central nervous system disorders. *Biomaterials.* **20** (4), 329-339 (1999).
19 Saltzman, W. M., Mak, M. W., Mahoney, M. J., Duenas, E. T. & Cleland, J. L. Intracranial Delivery of Recombinant Nerve Growth Factor: Release Kinetics and Protein Distribution for Three Delivery Systems. *Pharmaceutical Research.* **16** (2), 232-240 (1999).
20 Zhu, G., Mallery, S. R. & Schwendeman, S. P. Stabilization of proteins encapsulated in injectable poly (lactide- co-glycolide). *Nature Biotechnology.* **18** 52 (2000).
21 Anglin, E. J., Cheng, L., Freeman, W. R. & Sailor, M. J. Porous silicon in drug delivery devices and materials. *Advanced Drug Delivery Reviews.* **60** (11), 1266-1277 (2008).

22 Canham, L. T. *et al.* Derivatized mesoporous silicon with dramatically improved stability in simulated human blood plasma. *Advanced Materials.* **11** (18), 1505-1507 (1999).
23 Chen, F. *et al.* Organosilica Nanoparticles with an Intrinsic Secondary Amine: An Efficient and Reusable Adsorbent for Dyes. *ACS Applied Materials & Interfaces.* **9** (18), 15566-15576 (2017).
24 Godin, B. *et al.* Tailoring the degradation kinetics of mesoporous silicon structures through PEGylation. *Journal of Biomedical Materials Research Part A.* **94A** (4), 1236-1243 (2010).
25 Kempen, P. J. *et al.* Theranostic Mesoporous Silica Nanoparticles Biodegrade after Pro-Survival Drug Delivery and Ultrasound/Magnetic Resonance Imaging of Stem Cells. *Theranostics.* **5** (6), 631-642 (2015).
26 Low, S. P., Voelcker, N. H., Canham, L. T. & Williams, K. A. The biocompatibility of porous silicon in tissues of the eye. *Biomaterials.* **30** (15), 2873-2880 (2009).
27 Salonen, J., Kaukonen, A. M., Hirvonen, J. & Lehto, V.-P. Mesoporous silicon in drug delivery applications. *Journal of Pharmaceutical Sciences.* **97** (2), 632-653 (2008).
28 Tzur-Balter, A., Shatsberg, Z., Beckerman, M., Segal, E. & Artzi, N. Mechanism of erosion of nanostructured porous silicon drug carriers in neoplastic tissues. *Nature Communications.* **6** (2015).
29 Salonen, J. & Lehto, V.-P. Fabrication and chemical surface modification of mesoporous silicon for biomedical applications. *Chemical Engineering Journal.* **137** (1), 162-172 (2008).
30 Tzur-Balter, A., Massad-Ivanir, N. & Segal, E. Surface Engineered Porous Silicon-based Nanostructures for Cancer Therapy. *MRS Proceedings.* **1416** (2012).
31 Zilony, N. *et al.* Prolonged controlled delivery of nerve growth factor using porous silicon nanostructures. *Journal of Controlled Release.* **257** 51-59 (2017).
32 Young, M., Saide, J. D., Murphy, R. A. & Arnason, B. G. Molecular size of nerve growth factor in dilute solution. *Journal of Biological Chemistry.* **251** (2), 459-464 (1976).
33 Erickson, H. P. Size and Shape of Protein Molecules at the Nanometer Level Determined by Sedimentation, Gel Filtration, and Electron Microscopy. *Biological Procedures Online.* **11** (1), 32 (2009).
34 Jarvis, K. L., Barnes, T. J. & Prestidge, C. A. Thermal oxidation for controlling protein interactions with porous silicon. *Langmuir.* **26** (17), 14316-14322 (2010).
35 McInnes, S. J. *et al.* Surface engineering of porous silicon to optimise therapeutic antibody loading and release. *Journal of Materials Chemistry B.* **3** (20), 4123-4133 (2015).
36 Prestidge, C. A. *et al.* Loading and release of a model protein from porous silicon powders. *Physica Status Solidi (A).* **204** (10), 3361-3366 (2007).
37 Tzur-Balter, A., Young, J. M., Bonanno-Young, L. M. & Segal, E. Mathematical modeling of drug release from nanostructured porous Si: combining carrier erosion and hindered drug diffusion for predicting release kinetics. *Acta Biomaterialia.* **9** (9), 8346-8353 (2013).
38 Nieto, A. *et al.* Surface Engineering of Porous Silicon Microparticles for Intravitreal Sustained Delivery of Rapamycin. *Investigative Ophthalmology & Visual Science.* **56** (2), 1070-1080 (2015).
39 Warther, D. *et al.* Porous silicon based intravitreal platform for dual-drug loading and controlled release towards synergistic therapy. *Drug Delivery.* **25** (1), 1537-1545 (2018).
40 Li, W. *et al.* Tailoring Porous Silicon for Biomedical Applications: From Drug Delivery to

Cancer Immunotherapy. *Advanced Materials.* **30** (24), 1703740 (2018).
41 Yazdi, I. K. *et al.* Physicochemical properties affect the synthesis, controlled delivery, degradation and pharmacokinetics of inorganic nanoporous materials. *Nanomedicine.* **10** (19), 3057-3075 (2015).
42 Fry, N. L., Boss, G. R. & Sailor, M. J. Oxidation-Induced Trapping of Drugs in Porous Silicon Microparticles. *Chemistry of Materials.* **26** (8), 2758-2764 (2014).
43 Jarvis, K. L., Barnes, T. J. & Prestidge, C. A. Surface chemistry of porous silicon and implications for drug encapsulation and delivery applications. *Advances in Colloid and Interface Science.* **175** 25-38 (2012).
44 Salonen, J. & Mäkilä, E. Thermally Carbonized Porous Silicon and Its Recent Applications. *Advanced Materials.* **30** (24) (2018).
45 Wang, M. *et al.* Influence of Surface Chemistry on the Release of an Antibacterial Drug from Nanostructured Porous Silicon. *Langmuir.* **31** (22), 6179-6185 (2015).
46 Salonen, J. *et al.* Mesoporous silicon microparticles for oral drug delivery: loading and release of five model drugs. *Journal of Controlled Release.* **108** (2), 362-374 (2005).
47 Park, J.-H. *et al.* Biodegradable luminescent porous silicon nanoparticles for in vivo applications. *Nature Materials.* **8** (4), 331-336 (2009).
48 Johnson, P. J., Skornia, S. L., Stabenfeldt, S. E. & Willits, R. K. Maintaining bioactivity of NGF for controlled release from PLGA using PEG. *Journal of Biomedical Materials Research Part A.* **86** (2), 420-427 (2008).
49 Shefi, O. *et al.* Microtargeted gene silencing and ectopic expression in live embryos using biolistic delivery with a pneumatic capillary gun. *Journal of Neuroscience.* **26** (23), 6119-6123 (2006).
50 Zilony, N., Tzur-Balter, A., Segal, E. & Shefi, O. Bombarding Cancer: Biolistic Delivery of therapeutics using Porous Si Carriers. *Scientific Reports.* **3** 2499 (2013).
51 Zuidema, J. M., Provenza, C., Caliendo, T., Dutz, S. & Gilbert, R. J. Magnetic NGF-Releasing PLLA/Iron Oxide Nanoparticles Direct Extending Neurites and Preferentially Guide Neurites along Aligned Electrospun Microfibers. *ACS Chemical Neuroscience.* **6** (11), 1781-1788 (2015).
52 Baranes, K., Moshe, H., Alon, N., Schwartz, S. & Shefi, O. Neuronal Growth on l- and d-Cysteine Self-Assembled Monolayers Reveals Neuronal Chiral Sensitivity. *ACS Chemical Neuroscience.* **5** (5), 370-376 (2014).
53 Marcus, M. *et al.* Interactions of Neurons with Physical Environments. *Advanced Healthcare Materials.* **6** (15), 1700267 (2017).

Figure 1

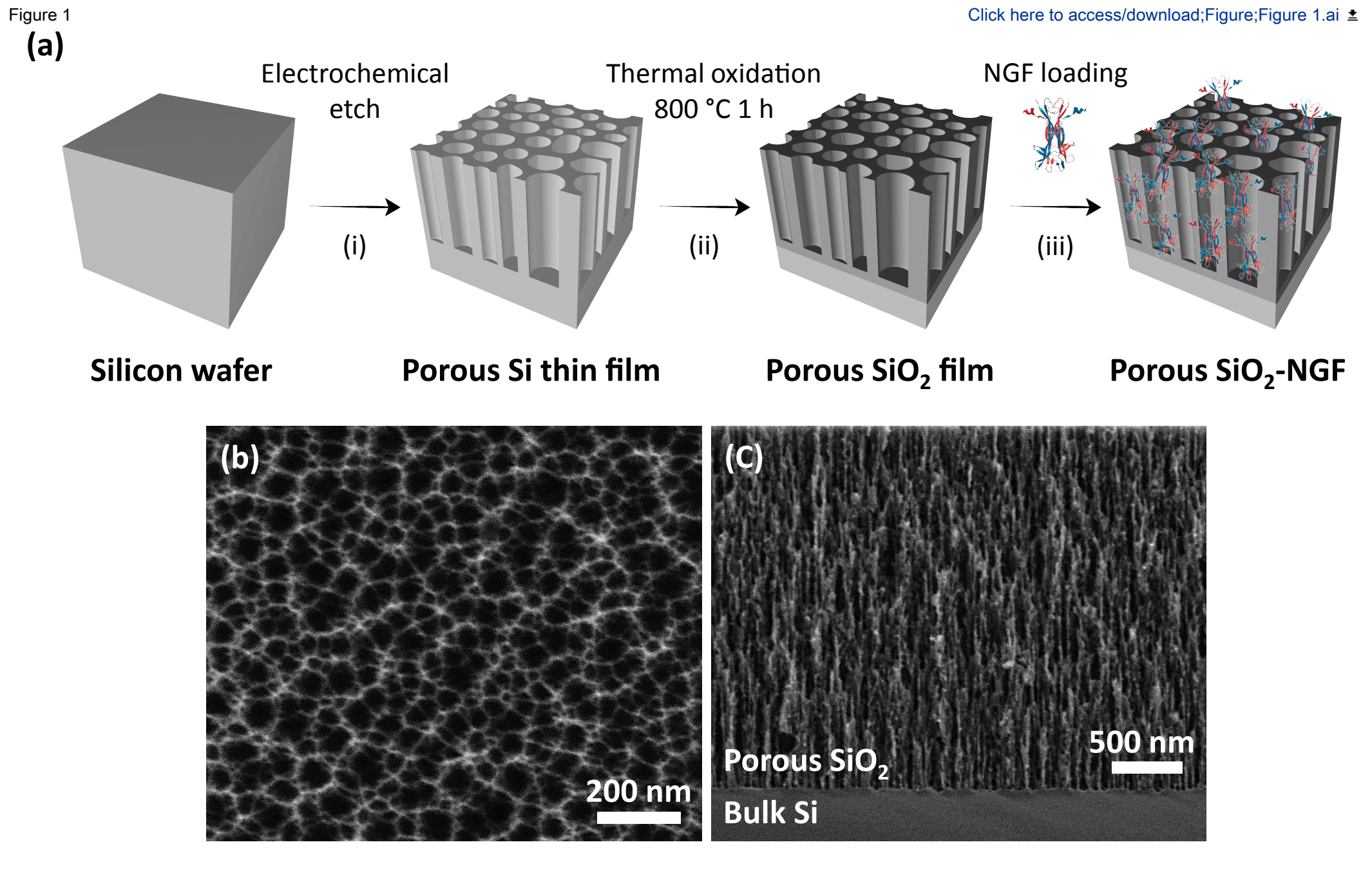


Figure 2

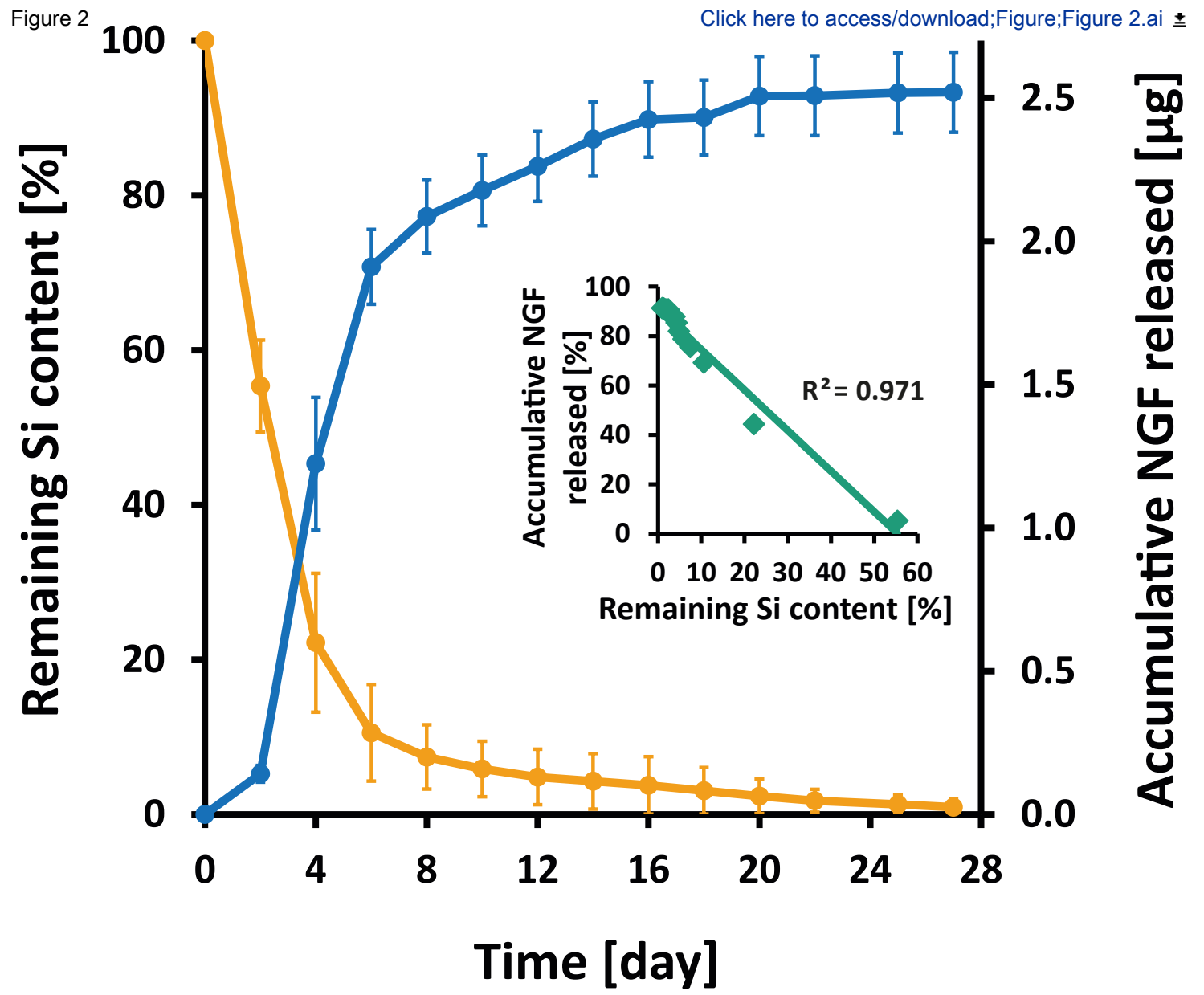


Figure 3

Click here to access/download;Figure;Figure 3.ai

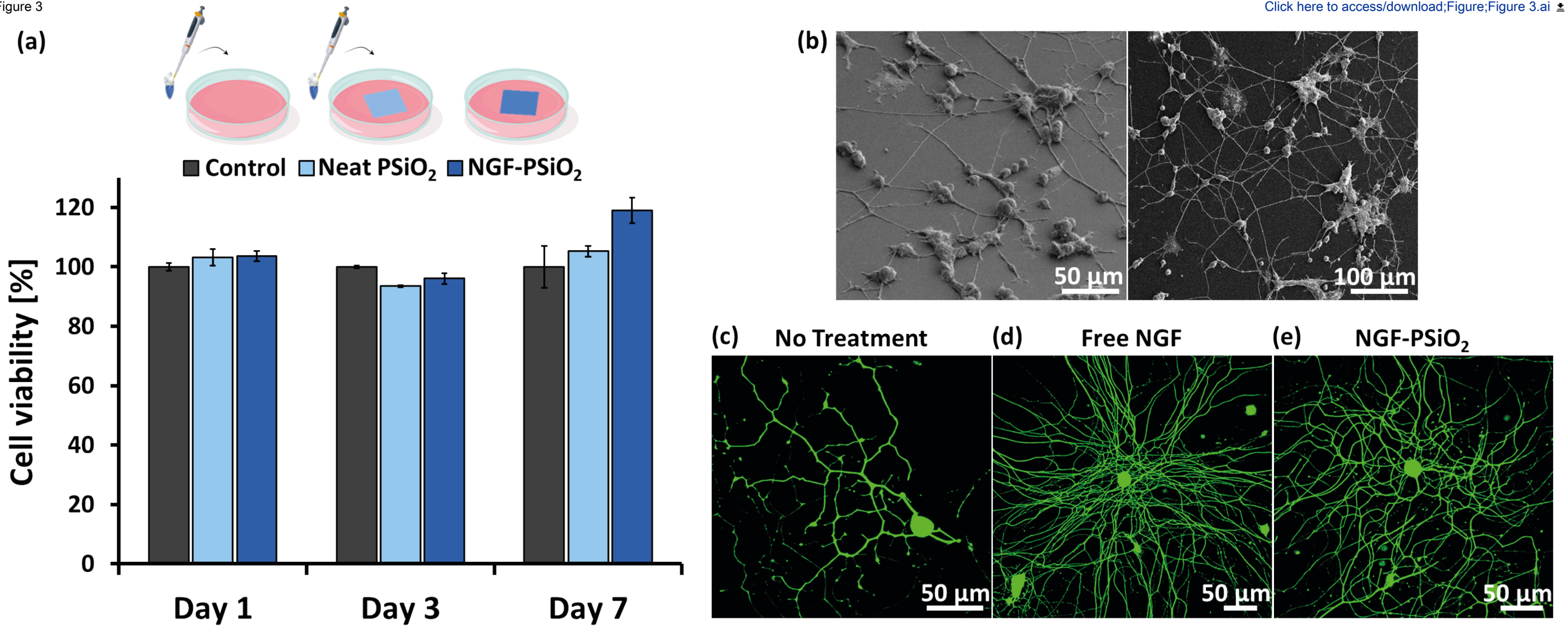


Figure 4

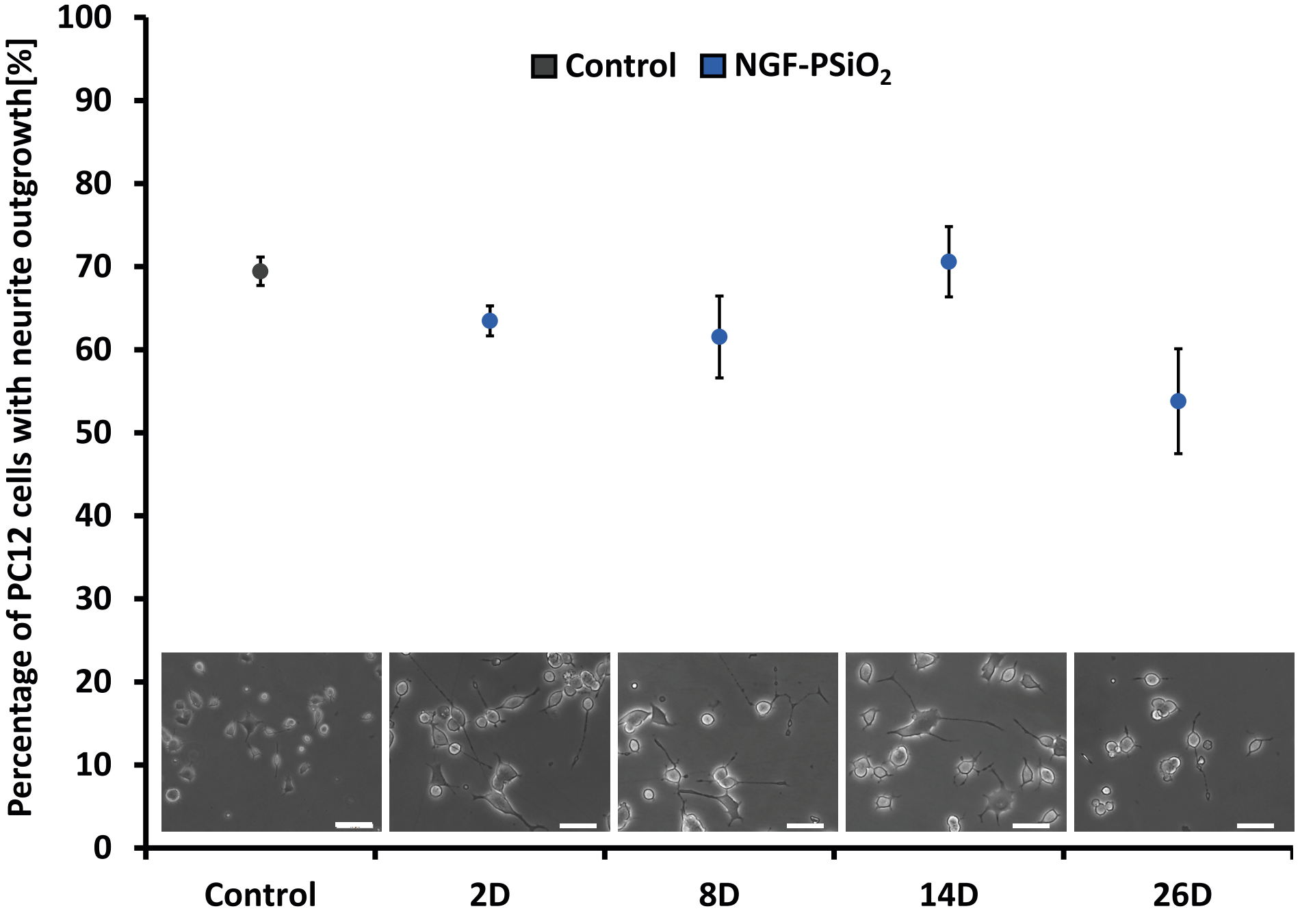


Figure 5

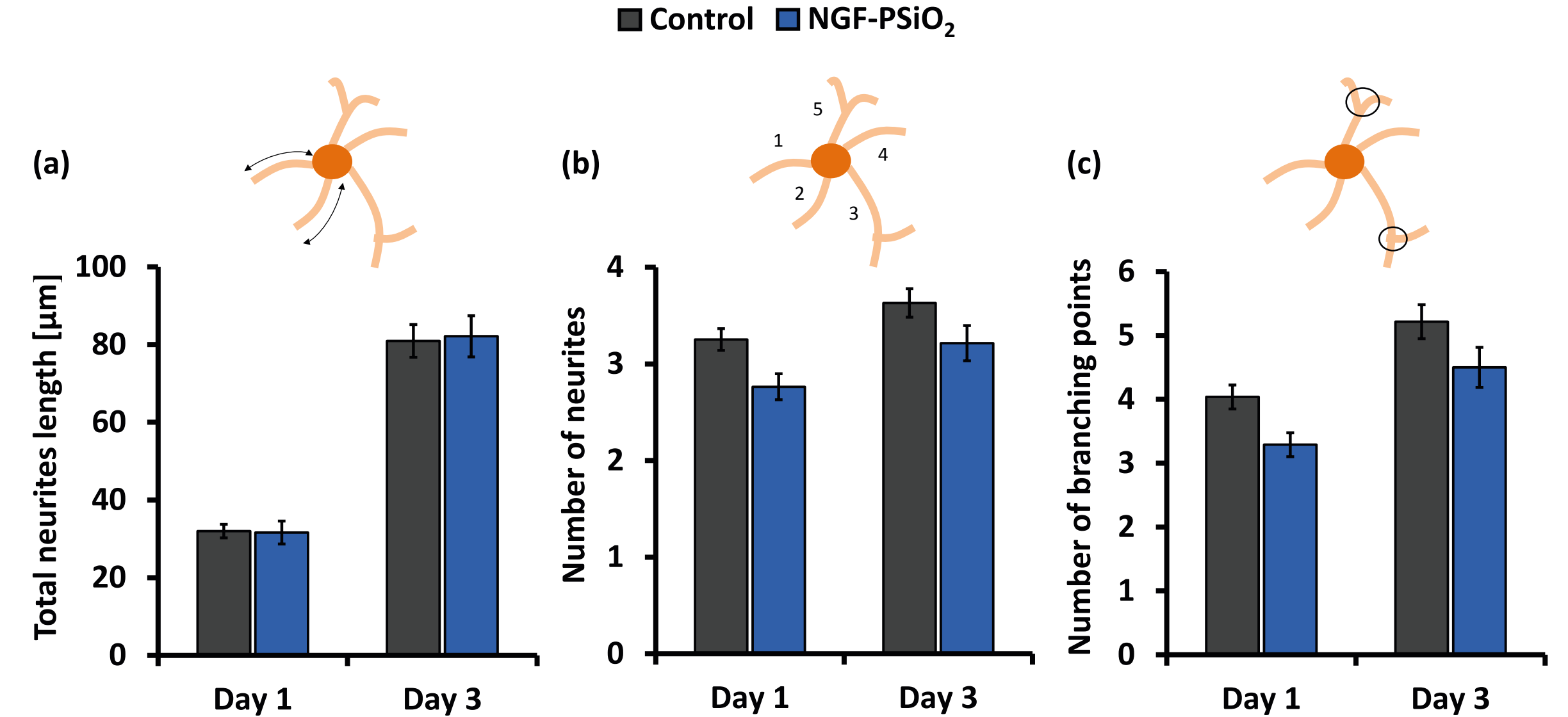


Table of Materials

Click here to access/download;Table of Materials;Rosenberg et al-Table of Materials.xls

Name of Material/ Equipment	Company	Catalog Number
Acetone	Gadot	830101375
Amphotericin	Biological Industries	03-028-1B
Aqueous HF (48%)	Merck	101513
AZ4533 photoresist	Metal Chem, Inc.	AZ4533
BSA fraction v	MP biomedicals	0216006950
BSA solution (10%)	Biological Industries	03-010-1B
Collagen type I	Corning Inc.	354236
Collagenase	Enco	LS004176
Collagen-coated plastic coverslips	NUNC Thermanox	1059846
D-(+)-glucose	Sigma-Aldrich Chemicals	G8170
Dispase-II	Sigma-Aldrich Chemicals	4942078001
Donkey anti mouse IgG H&L conjugated Alexa Fluor 488	Abcam	ab150073
Ethanol absolute (99.9%)	Merck	818760
FBS	Biological Industries	04-121-1A
Formaldehyde/glutaraldehyde (2.5%) in 0.1 M sodium cacodylate	Electron Microscopy Sciences	15949
Freon	Sigma-Aldrich Chemicals	613894
Guanidine-HCl	Sigma-Aldrich Chemicals	G7294

Ham's F-12 nutrient mixture	Thermo Scientific	11765054
HBSS	Thermo Scientific	14185-045
HEPES (1M)	Thermo Scientific	15630-056
HS	Biological Industries	04-124-1A
Human β-NGF ELISA Development Kit	Peprotech	900-K60
Immumount solution	Thermo Scientific	9990402
L-15 medium	Sigma-Aldrich Chemicals	L5520
Laminin	Thermo Scientific	23017015
L-glutamine	Biological Industries	03-020-1A
Mouse anti neurofilament H (NF-H) (phosphorylated antibody) antibody	BiolLegend	SMI31P
Murine β-NGF	Peprotech	450-34-20
Normal donkey serum (NDS)	Sigma-Aldrich Chemicals	G9023
Papain	Sigma-Aldrich Chemicals	p-4762
Paraformaldehyde 16% solution	Electron Microscopy Sciences	BN15710
PBS (pH 7.4)		

PBS X10	Biological Industries	02-020-1A
PC12 cell line	ATCC	CRL-1721
Penicillin–streptomycin	Biological Industries	03-032-1B
Percoll	Sigma-Aldrich Chemicals	p1644
Poly-L-lysine	Sigma-Aldrich Chemicals	P4832
PrestoBlue reagent	Thermo Scientific	A13261
RPMI medium	Biological Industries	01-100-1A
Si wafer	Siltronix Corp.	
Sodium azide	Sigma-Aldrich Chemicals	S2002
Sodium hydroxide (NaOH)	Sigma-Aldrich Chemicals	S8045
Tannic acid	Sigma-Aldrich Chemicals	403040
Triton X-100	Chem-Impex International Inc.	1279

Comments/Description

prepared by dissolving 10 mM Na2HPO4, 1.8 mM KH2PO4, 137 mM

Highly-B-doped, p-type, 0.00095
Ω-cm resistivity, <100> oriented

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Designing Porous Silicon Films as Carriers of Nerve Growth Factor for Promoting Differentiation and Outgrowth of PC12 Cells and Dorsal Root Ganglion

Author(s): Michal Rosenberg, Neta Zilony, Orit Shefi and Ester Segal

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/author) via: [ ] Standard Access [x] Open Access

Item 2 (check one box):

[x] The Author is NOT a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	Ester Segal
Department:	Biotechnology and Food Engineering
Institution:	Technion
Article Title:	Porous Silicon for Controlled Release of Nerve Growth Factor: Promoting Neuronal Differentiation and Outgrowth of PC12 Cells and Dorsal Root Ganglion
Signature:	Digitally signed by Ester Segal Date: 2018.10.22 13:12:29 +03'00'
Date:	22/10/18

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

Technion
Israel Institute of Technology

Ester Segal, Ph.D.
Associate Professor
Department of Biotechnology and Food Engineering
Russell Berrie Nanotechnology Institute
Technion, Haifa 32000, Israel
TEL 972-4-8295071
Email esegal@technion.ac.il
Website: http://segallab.technion.ac.il/

22/10/2018

To: Dr. Wu, Review Editor

JoVE

Dear Dr. Bing Wu,

We would like to thank you for sending out our manuscript for review and providing us a rapid and thorough evaluation of our work. We appreciate the referees' constructive comments and your willingness to further consider our manuscript.

We have made the appropriate changes as requested by all three reviewers and believe that this manuscript has greatly benefited as the result of these changes. We also addressed all editorial issues. The revised manuscript is attached along with our detailed response to the reviewers' questions and comments. We have also included (in a separate file) the revised manuscript in which our changes are marked in red to ease the review.

We believe that the manuscript is ready for further consideration in *JoVE*.

Sincerely,

Ester Segal

Detailed response to editorial comments and reviewers' comments:

Editorial comments:

Changes to be made by the author(s) regarding the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Answer: We thank the editor for his careful review and we have thoroughly proofread the manuscript.

2. Please revise lines 45-46, 64-65, and 68-69 to avoid previously published text.

Answer: The mentioned lines have been rewritten and rephrased.

3. Please revise the title to avoid punctuation.

Answer: The title has been changed to avoid punctuation.

4. Please remove all commercial language from your manuscript and use generic terms instead: AZ4533, percoll, etc.

Answer: In line 129, AZ4533 was replaced with "a positive thick". In line 305, percoll was replaced with "silica-based colloidal medium for cell separation by density gradient centrifugation".

5. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below:

2.4: Please ensure that the protocol here can stand alone. As currently written, users must refer to another protocol in order to complete this protocol. Please describe how to determine NGF content.

Answer: Step 2.4 in now thoroughly detailed in a new section that was added to the revised manuscript (section 3. Quantification of NGF loading using NGF ELISA kit), see page 4.

3.4: Please describe how to perform ICP-AES analysis. We need more specific details for filming.

Answer: Step 3.4 is now described in details in a new section that was added to the revised manuscript (section 5. Quantification of in vitro Si erosion by inductively coupled plasma atomic emission spectroscopy (ICP-AES)), see pages 5-6.

4.2.1: Please specify how to isolate DRGs from mice.

Answer: This step is now elaborated and detailed in 5 new steps that were added to the revised manuscript, see pages 7.

4.2.2: How to clean the surrounding connective tissues?

Answer: These details are now specified in step 6.2.6.

6. Line 211: Please move the ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Answer: We thank the editor for this comment and we have moved the ethics statement accordingly, before the numbered protocol steps.

7. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Answer: We have highlighted 2.75 pages of the revised protocol to identifie the essential steps of the protocol for the video.

8. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

Answer: The highlighted sentences in the revised manuscript follow the instructions above.

9. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Answer: We have carefully followed these instructions in the revised highlighted protocol.

10. Figures 2, 4 and 5: Please define error bars in the figure legend.

Answer: We have added definitions for the error bars to the captions of Figures 2, 4, and 5. In all of them the error bars represent SD, n=3.

11. Figure 3b: Please describe the left and right panels.

Answer: We have added description of the left and right panels to the caption of Figure 3.

Reviewers' comments:

Reviewer #1:

Manuscript summary:

Fabrication of porous silicon (PSi) film as a degradable carrier for nerve growth factor (NGF) release is reported. The PSi carrier was designed to exhibit high loading capacity of NGF, and sustained release was examined in vitro for four weeks. Differentiation of PC12 cells and DRG neuronal cells in NGF-releasing condition was characterized and compared to conventional free NGF administration. The results showed comparable neurite

initiation to the free NGF administration, indicating feasibility of the PSi carrier as a long-term implant for NGF release to treat neurodegenerative diseases. Because of the biocompatibility and biodegradability of PSi, this platform can be a promising delivery system for long-term NGF or other growth factor supply. This report include essential information to fabricate the PSi-based NGF releasing system with step-by-step protocol, therefore the experiment can be readily reproduced by others. Experimental and analytical details are sufficiently described in the present form. This report will be a good reference in the field, and attract much attention to the general readership of the Journal of Visualized Experiment. Therefore, it is highly recommended to be published in the Journal of Visualized Experiment after revision of a few minor concerns as following.

We thank the reviewer for his/her positive evaluation of our manuscript and have carefully revised it based on his/her feedback.

Minor concerns:

1) In the Protocol 1.1, the kind of dopants (e.g., boron) should be noted specifically.

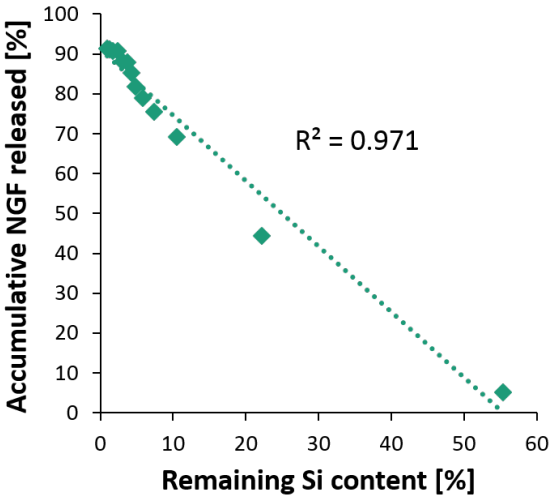
Answer: The dopant type (Boron) is now specified in step 1.1 of the revised protocol.

2) In the Protocol 1.2, 1.9, and 1.10, heating (10 C per min?) and cooling (natural cooling?) rate should be stated.

Answer: We have added the details regarding the heating and cooling rates for step 1.2, 1.9, 1.10 in the revised protocol.

3) It would be helpful to show strong correlation of NGF release with Si degradation profile by plotting NGF release as a function of remaining Si content over time in Figure 2.

Answer: We thank the reviewer for this constructive comment. Please see attached below a graph presenting the excellent correlation between the NGF release and the remaining Si content. We have included this new graph as an inset in Figure 2 and have addressed it in the revised manuscript, see page 10.



4) It would be helpful if additional information about the a few more set of etching condition, pore size, and NGF loading capacity is included.

Answer: In this work we have focused on a specific nanostructured PSi film, which was found to yield the maximal NGF loading efficacy. A prior thorough optimization study, we have investigated the correlation between the anodization conditions, resulting nanostructure characteristics, and NGF loading and release. Please see attached below unpublished data of the prior optimization work performed. Table S1 summarizes the different etching conditions examined and Figure S1 presents NGF release profiles of the corresponding $PSiO_2$ carriers. Condition set no. 4 in Table S1 yielded the highest values of accumulative NGF release (Figure S1) compared to the other $PSiO_2$ carriers examined, and this was one of the reasons for focusing on this specific set of etching conditions. We believe that these results are beyond the scope of this paper, which focuses on the controlled release of NGF and its bioactivity from PSi carriers.

Table S1. Etching parameters

Current density [mA/cm²]	Etching time [sec]
50	101
100	51
150	34
250	20

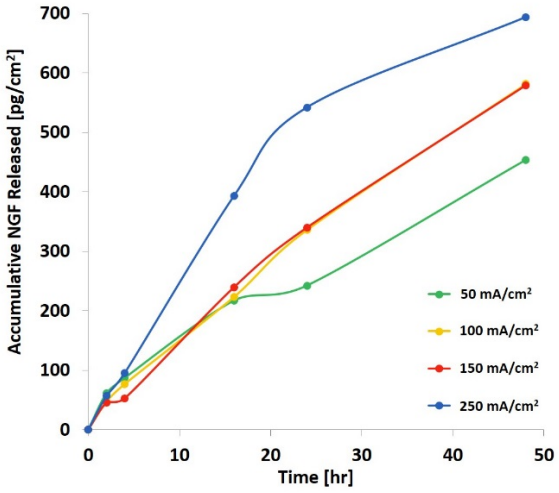


Figure S1: NGF release profiles of the different NGF-loaded $PSiO_2$ carriers

5) As authors mentioned in the manuscript, the release of NGF payload is affected by two

pathway: out-diffusion and silicon scaffold degradation. Therefore, it would be very nice if authors state any information to distinguish each kinetics or suggest any methods for further analyses.

Answer: We thank the reviewer for this important comment. The mentioned concept is elaborated in previous works by our group and others and we now refer the readers to relevant references in the discussion section and expand the discussion, see page 12. The two mechanisms, i.e., out-diffusion and degradation of the Si scaffold, occur simultaneously for oxidized PSi and it is difficult to clearly differentiate and identify the amount of drug released due to passive diffusion versus the amount of drug released due to scaffold degradation.

6) The release kinetics of the NGF payload is not a typical curve of 'sustained release'. However, authors claim that a sustained release of NGF without burst release is attained for 1 month. It is recommended to discuss it carefully. The release curve still has a sort of burst release at the very beginning (within 6 days).

Answer: We agree with the reviewer and accordingly have added a clarification and better description of the release kinetics in the revised manuscript, page 10.

Reviewer #2:

Manuscript Summary:

This is a revision of a methods paper to JoVE. I see some highlighted text suggesting that the paper has been revised although I do not see the original reviewer comments. This is suitable for publication after these minor issues are addressed.

1. 2.1. Is there need for BSA as a carrier protein? Might this improve efficacy?

Answer: We thank the reviewer for the constructive comment. Many reports utilize BSA as a carrier protein in order to stabilize the NGF and prevent its denaturation and loss of biological activity during the encapsulation process. In our work, NGF loading into the carriers is carried out at room temperature without using organic solvents and thus the entrapment within the carrier system does not impair the bioactivity of the protein. Moreover, co-loading of NGF and BSA may result in a lower loading efficacy compared to loading of NGF alone as the available volume for accommodation of NGF within the host is reduced when loading both of the proteins. However, adding BSA can be advantageous in terms of sustaining NGF release from the carriers for an extended time period. Numerous studies have demonstrated the use of BSA as a carrier protein to modulate the release rate of NGF; incorporating BSA within the carrier matrix or co-loading of BSA and NGF have been shown to significantly extend the NGF release period[1-3].

1 Valmikinathan, C. M., Defroda, S. & Yu, X. Polycaprolactone and bovine serum albumin based nanofibers for controlled release of nerve growth factor. *Biomacromolecules.* **10** (5), 1084-1089, (2009).
2 Xu, X. *et al.* Peripheral nerve regeneration with sustained release of poly(phosphoester) microencapsulated nerve growth factor within nerve guide conduits. *Biomaterials.* **24** (13), 2405-2412, (2003).
3 Xu, X. *et al.* Polyphosphoester microspheres for sustained release of biologically active nerve growth factor. *Biomaterials.* **23** (17), 3765-3772, (2002).

2. 2.4. Can you recommend a specific manufacturer? Lots of bad ELISA kits.

Answer: The manufacturer of the NGF ELISA kit used in this work is PeproTech, as cited in the table of materials. Detailed information regarding the use of the kit can be found in step 3 in the revised protocol section.

3. 4.1.3. Recommended concentration of cells and seeding density?

Answer: We have added the details regarding recommended concentration of cells and seeding density in step 6.1.3 in the revised protocol.

4. Missing citations to Si-based drug delivery:

a. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4377731/
b. https://pubs.acs.org/doi/full/10.1021/acsami.7b04181

Answer: We thank the reviewer suggesting these references and we now properly cite them is the revised introduction of the manuscript, see references 23 and 25.

Reviewer #3:

The work by Segal's group describes the fabrication of nanostructured PSi films for continuous and prolonged drug release, providing bioactivity of NGF released from the PSi carriers. The studied is well conducted, very well presented and very interesting for the broad audience of this journal. Therefore, I'd recommend acceptance of this work in its present form.

We thank the referee for his positive evaluation of our work and supporting its publication.

TITLE:

Designing Porous Silicon Films as Carriers of Nerve Growth Factor for Promoting Differentiation and Outgrowth of PC12 Cells and Dorsal Root Ganglion

AUTHORS AND AFFILIATIONS:

Michal Rosenberg[1,], Neta Zilony[2,3,*], Orit Shefi[2,3] and Ester Segal[1,4]

[1]Department of Biotechnology and Food Engineering, Technion – Israel Institute of Technology, Technion City, Haifa 3200003, Israel

[2]Faculty of Engineering, Bar-Ilan University, Ramat-Gan 5290002, Israel

[3]Bar-Ilan Institute of Nanotechnologies and Advanced Materials, Ramat-Gan, 5290002, Israel

[4]Russell Berrie Nanotechnology Institute, Technion – Israel Institute of Technology, Technion City, Haifa 3200003, Israel

[*]The authors contributed equally to this work

Corresponding Authors:

Ester Segal

esegal@technion.ac.il

Tel: (972)-4-8295071

Orit Shefi

orit.shefi@biu.ac.il

Tel: (972)-3-5317079

Email Addresses of Co-authors**:**

Neta Zilony (neta.zilony@biu.ac.il)

Michal Rosenberg (rosenbem@campus.technion.ac.il)

KEYWORDS:

Nerve Growth Factor; Porous Silicon; PC12; Dorsal Root Ganglia; Controlled Release; Neuronal Differentiation.

SUMMARY:

In this work, we design and fabricate nanostructured porous silicon (PSi) films as degradable carriers for nerve growth factor (NGF). Neuronal differentiation and outgrowth of PC12 cells and mice dorsal root ganglion (DRG) neurons are characterized upon treatment with the NGF-loaded PSi carriers.

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 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 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 of long-term implants for NGF release with a therapeutic potential for neurodegenerative diseases.

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[3-6]. 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[10-17]. 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[21-28]. 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:

1. Fabrication of oxidized PSi ($PSiO_2$) carriers

1.1. Cut a Si wafer (single side polished on the <100> face and heavily Boron-doped, p-type, 0.95 mΩ·cm) into 1.5 × 1.5 cm samples using a diamond-tipped pen.

1.2. Oxidize the Si samples in a tube furnace at 400 °C for 2 h in ambient air (heating rate: 25 °C/min, natural cooling).

1.3. Immerse the Si samples in a solution of aqueous hydrofluoric acid (HF) (48%), ddH_2O and ethanol (99.9%) (1:1:3 v/v) for 5 min; then rinse the samples with ethanol for 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.

1.4. Mount the Si sample in a Teflon etching cell, using a strip of aluminum foil as a back-contact and a platinum coil as the counter electrode.

1.5. 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 for three times and dry under a nitrogen stream.

1.6. Dissolve the freshly-etched porous layer in an aqueous NaOH solution (0.1 M) for 2 min; then rinse with ethanol for three times and dry under a nitrogen stream.

1.7. 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 for three times and dry under a nitrogen stream.

1.8. 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 for three times and dry under a nitrogen stream.

1.9. 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.

1.10. Spin-coat the $PSiO_2$ samples with a positive thick photoresist at 4000 rpm for 1 min; then bake the coated samples at 90 °C for 2 min (heating rate: 5 °C/min, natural cooling).

1.11. Dice the $PSiO_2$ samples into 8 × 8 mm samples using a dicing saw.

1.12. 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

2.1. 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.

2.2. Add 52 µL of the loading solution on top of the $PSiO_2$ sample and incubate for 2 h at room temperature in a capped dish.

Note: Maintain high humidity in the dish to prevent drying up of the solution during incubation.

2.3. Collect the solution on 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 risk of drying and denaturation of the protein.

3. Quantification of NGF loading using NGF ELISA kit

3.1. 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 room temperature.

3.2. Aspirate the wells to remove liquid and wash the plate 4 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.

3.3. Add 300 µL of block buffer (1% bovine serum albumin (BSA) in PBS) to each well and incubate for at least 1 h at room temperature; then aspirate and wash the plate 4 times as described in step 3.2.

3.4. 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.

3.5. 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 kit (16-1000 pg/mL).

3.6. Add 100 µL of the diluted samples and calibration curve samples to each well in triplicate and incubate at room temperature for 2 h; then aspirate and wash the plate 4 times as described in step 3.2.

3.7. Add 100 µL of 1 µg/mL detection antibody (biotinylated rabbit anti-hβ-NGF) to each well and incubate at room temperature for 2 h; then aspirate and wash the plate 4 times as described in step 3.2.

3.8. Dilute Avidin-Horseradish Peroxidase (HRP) 1:2000 in diluent, add 100 µL per well and incubate for 30 min at room temperature; then aspirate and wash the plate 4 times as described in step 3.2.

3.9. Add 100 µL of ABTS liquid substrate solution to each well and incubate 25 min at room temperature for color development; Measure absorbance at 405 nm with wavelength correction set at 650 nm using a microplate reader.

3.10. Determine NGF concentration in both the loading solution and the collected post-loading solution based on the calibration curve.

3.11. 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 $PSiO_2$

4.1. 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.

4.2. 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.

4.3. Use NGF ELISA kit 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.

4.4. 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)

5.1. 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.

5.2. Monitor Si atomic emission peaks at 212.4, 251.6 and 288.2 nm for the diluted samples.

5.3. Determine Si concentration in the samples based on a calibration curve.

5.4. 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

6.1. Rat pheochromocytoma (PC12) cell culture

6.1.1. Prepare 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.

6.1.2. Prepare differentiation medium by adding 1 % HS, 1 % L-glutamine, 1 % penicillin streptomycin and 0.2 % amphotericin to RPMI medium.

6.1.3. 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.

6.1.4. To generate differentiated PC12 cell culture, transfer the cell suspension to a centrifuge tube; centrifuge cells at 200 x g for 8 min at room temperature and discard the supernatant.

6.1.5. Suspend the cells in 5 mL of fresh basic growth medium and re-centrifuge the cells at 200 x g for 5 min at room temperature; discard the supernatant and resuspend the cell pellet in 3 mL of basic growth medium.

6.1.6. To separate cell clusters, aspirate the cells 10 times using a 23 G syringe.

6.1.7. Count the cells using a hemocytometer cell counter and seed 10^4 cells/1 cm^2 working area on collagen type I coated plates in the presence of differentiation medium.

6.1.8. 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.

6.1.9. Renew the differentiation medium every 2 days.

6.1.10. 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 absorbance at 490 nm using a spectrophotometer.

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

6.2. Mice dorsal root ganglia (DRG) cell culture

6.2.1. 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.

6.2.2. Cut the base of the skull and the abdominal wall muscles till the spinal cord is exposed.

6.2.3. Remove the spinal column by making a cut at the level of the femurs and clean the surrounding tissues.

6.2.4. Cut the column into three pieces; then cut each piece in the midline to generate two halves and peel out the spinal cord.

6.2.5. Under the binocular, extract all DRG ganglia and immerse them in a cold Hank's balanced salt solution (HBSS).

6.2.6. Clean the surrounding connective tissues by pining the DRGs on a Sylguard petri dish and gently remove residual meninges under the binocular.

6.2.7. Dissociate the DRG cells by incubating them for 20 min in 1000 U of papain.

6.2.8. Centrifuge the cells for 4 min at 400 x g; discard the supernatant and keep the cell pellet.

6.2.9. Resuspend the pellet in a 10 mg/mL collagenase and 12 mg/mL dispase-II solution and incubate for 20 min at 37 °C.

6.2.10. Centrifuge the cells for 2 min at 400 x g; discard the supernatant and keep the cell pellet.

6.2.11. 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.

6.2.12. 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 1000 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.

6.2.13. Resuspend the cell pellet in 2 mL of F-12 medium; centrifuge for 2 min at 1000 x g; discard the supernatant and resuspend the pellet in 1 mL of F-12 medium.

6.2.14. 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.

6.2.15. Gently add the NGF-loaded $PSiO_2$ carrier or fresh murine β-NGF (50 ng/mL) per plate.

6.2.16. After 2 days, fix the cell culture by incubating it with a 4 % paraformaldehyde (PFA) solution for 15 min at room temperature; immunostain the cell culture using a common immunofluorescence staining procedure[31].

6.2.17. Image the neuronal cell culture using a confocal microscope.

7. Cell differentiation analysis

7.1. PC12 cells differentiation percentage in the presence of NGF-loaded $PSiO_2$ carriers

7.1.1. Incubate the NGF-loaded $PSiO_2$ carriers in 2 mL of differentiation medium, in a humidified incubator at 37 °C containing 5 % CO_2.

7.1.2. 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.

7.1.3. Seed PC12 cells in 12-well collagen-coated plates as instructed in section 4.1 (4.1.3-4.1.6).

7.1.4. 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).

7.1.5. After 24 h, image the cells using a light microscope.

7.1.6. 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.

7.2. Morphometric analysis of differentiated PC12 cells

7.2.1. 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.

7.2.2. Download the image processing program NeuronJ, an ImageJ plug-in, which enables a semi-automatic neurite tracing and length measuring.

7.2.3. Convert the image file to an 8-bit format and measure pixel to micron ratio using the image scale bar.

7.2.4. Set the scale using the Analyze/Set Scale command and measure the length of each neurite.

7.2.5. 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 micron. The measured neurite lengths may be widely distributed.

REPRESENTATIVE RESULTS:

Oxidized PSi films are fabricated as described in the Protocol Text section. 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 kit 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 kit. 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 is 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 Text section. 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.

In order 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 AND TABLE LEGENDS:

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.

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.

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.

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.

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.

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*.

Critical steps in the protocol:

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[34-36]. 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.

Modifications and troubleshooting of the method:

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 requited 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[35,42-45]. 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.

Limitations of the method

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 Text section, 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.

Significance with respect to existing methods

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[10-12,15-17]. 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 room temperature 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.

Future applications

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 enables 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.

ACKNOWLEDGMENTS:

MS and ES acknowledge the core services and support from the Lorry I. Lokey Center for Life Science and Engineering and the financial support of the Russell Berrie Nanotechnology Institute at the Technion.

DISCLOSURES:

The authors declare no competing financial interests.

REFERENCES

1 Wiesmann, C. & De Vos, A. Nerve growth factor: structure and function. *Cellular and Molecular Life Sciences.* **58** (5), 748-759, (2001).

2 Dreyfus, C. F. Effects of nerve growth factor on cholinergic brain neurons. *Trends in pharmacological sciences.* **10** (4), 145-149, (1989).

3 Eriksdotter Jönhagen, M. *et al.* Intracerebroventricular infusion of nerve growth factor in three patients with Alzheimer's disease. *Dementia and geriatric cognitive disorders.* **9** (5), 246-257, (1998).

4 Eriksdotter-Jönhagen, M. *et al.* Encapsulated cell biodelivery of nerve growth factor to the basal forebrain in patients with Alzheimer's disease. *Dementia and geriatric cognitive disorders.* **33** (1), 18-28, (2012).

5 Eyjolfsdottir, H. *et al.* Targeted delivery of nerve growth factor to the cholinergic basal forebrain of Alzheimer's disease patients: application of a second-generation encapsulated cell biodelivery device. *Alzheimer's Research & Therapy.* **8** (1), 30, (2016).

6 Tuszynski, M. H. *et al.* A phase 1 clinical trial of nerve growth factor gene therapy for Alzheimer disease. *Nature medicine.* **11** (5), 551-555, (2005).

7 Pan, W., Banks, W. A. & Kastin, A. J. Permeability of the blood–brain barrier to neurotrophins. *Brain research.* **788** (1), 87-94, (1998).

8 Thorne, R. G. & Frey, W. H. Delivery of neurotrophic factors to the central nervous system. *Clinical pharmacokinetics.* **40** (12), 907-946, (2001).

9 Tria, M. A., Fusco, M., Vantini, G. & Mariot, R. Pharmacokinetics of nerve growth factor (NGF) following different routes of administration to adult rats. *Experimental neurology.* **127** (2), 178-183, (1994).

10 Bhang, S. H. *et al.* The effect of the controlled release of nerve growth factor from collagen gel on the efficiency of neural cell culture. *Biomaterials.* **30** (1), 126-132, (2009).

11 Camarata, P. J., Suryanarayanan, R., Turner, D. A., Parker, R. G. & Ebner, T. J. Sustained release of nerve growth factor from biodegradable polymer microspheres. *Neurosurgery.* **30** (3), 313-319, (1992).

12 Cooper, A., Bhattarai, N. & Zhang, M. Fabrication and cellular compatibility of aligned chitosan–PCL fibers for nerve tissue regeneration. *Carbohydrate Polymers.* **85** (1), 149-156, (2011).

13 Marcus, M. *et al.* Iron oxide nanoparticles for neuronal cell applications: uptake study and magnetic manipulations. *Journal of Nanobiotechnology.* **14** (1), 37, (2016).

14 Marcus, M., Skaat, H., Alon, N., Margel, S. & Shefi, O. NGF-conjugated iron oxide nanoparticles promote differentiation and outgrowth of PC12 cells. *Nanoscale.* **7** (3), 1058-1066, (2015).

15 Sridhar, R. *et al.* Electrosprayed nanoparticles and electrospun nanofibers based on natural materials: applications in tissue regeneration, drug delivery and pharmaceuticals. *Chemical Society Reviews.* **44** (3), 790-814, (2015).

16 Valmikinathan, C. M., Defroda, S. & Yu, X. Polycaprolactone and bovine serum albumin based nanofibers for controlled release of nerve growth factor. *Biomacromolecules.* **10** (5), 1084-1089, (2009).

17 Xu, X. *et al.* Polyphosphoester microspheres for sustained release of biologically active nerve growth factor. *Biomaterials.* **23** (17), 3765-3772, (2002).

18 Cao, X. & Shoichet, M. S. Delivering neuroactive molecules from biodegradable microspheres for application in central nervous system disorders. *Biomaterials.* **20** (4), 329-339, (1999).

19 Saltzman, W. M., Mak, M. W., Mahoney, M. J., Duenas, E. T. & Cleland, J. L. Intracranial Delivery of Recombinant Nerve Growth Factor: Release Kinetics and Protein Distribution for Three Delivery Systems. *Pharmaceutical research.* **16** (2), 232-240, (1999).

20 Zhu, G., Mallery, S. R. & Schwendeman, S. P. Stabilization of proteins encapsulated in injectable poly (lactide- co-glycolide). *Nature biotechnology.* **18** 52, (2000).

21 Anglin, E. J., Cheng, L., Freeman, W. R. & Sailor, M. J. Porous silicon in drug delivery devices and materials. *Advanced drug delivery reviews.* **60** (11), 1266-1277, (2008).

22 Canham, L. T. *et al.* Derivatized mesoporous silicon with dramatically improved stability in simulated human blood plasma. *Advanced Materials.* **11** (18), 1505-1507, (1999).

23 Chen, F. *et al.* Organosilica Nanoparticles with an Intrinsic Secondary Amine: An Efficient and Reusable Adsorbent for Dyes. *ACS Applied Materials & Interfaces.* **9** (18), 15566-15576, (2017).

24 Godin, B. *et al.* Tailoring the degradation kinetics of mesoporous silicon structures through PEGylation. *Journal of Biomedical Materials Research Part A.* **94A** (4), 1236-1243, (2010).

25 Kempen, P. J. *et al.* Theranostic Mesoporous Silica Nanoparticles Biodegrade after Pro-Survival Drug Delivery and Ultrasound/Magnetic Resonance Imaging of Stem Cells. *Theranostics.* **5** (6), 631-642, (2015).

26 Low, S. P., Voelcker, N. H., Canham, L. T. & Williams, K. A. The biocompatibility of porous silicon in tissues of the eye. *Biomaterials.* **30** (15), 2873-2880, (2009).

27 Salonen, J., Kaukonen, A. M., Hirvonen, J. & Lehto, V.-P. Mesoporous silicon in drug delivery applications. *Journal of Pharmaceutical Sciences.* **97** (2), 632-653, (2008).

28 Tzur-Balter, A., Shatsberg, Z., Beckerman, M., Segal, E. & Artzi, N. Mechanism of erosion of nanostructured porous silicon drug carriers in neoplastic tissues. *Nature communications.* **6**, (2015).

29 Salonen, J. & Lehto, V.-P. Fabrication and chemical surface modification of mesoporous silicon for biomedical applications. *Chemical Engineering Journal.* **137** (1), 162-172, (2008).

30 Tzur-Balter, A., Massad-Ivanir, N. & Segal, E. Surface Engineered Porous Silicon-based Nanostructures for Cancer Therapy. *MRS Proceedings.* **1416**, (2012).

31 Zilony, N. *et al.* Prolonged controlled delivery of nerve growth factor using porous silicon nanostructures. *Journal of Controlled Release.* **257** 51-59, (2017).

32 Young, M., Saide, J. D., Murphy, R. A. & Arnason, B. G. Molecular size of nerve growth factor in dilute solution. *Journal of Biological Chemistry.* **251** (2), 459-464, (1976).

33 Erickson, H. P. Size and Shape of Protein Molecules at the Nanometer Level Determined by Sedimentation, Gel Filtration, and Electron Microscopy. *Biological procedures online.* **11** (1), 32, (2009).

34 Jarvis, K. L., Barnes, T. J. & Prestidge, C. A. Thermal oxidation for controlling protein interactions with porous silicon. *Langmuir.* **26** (17), 14316-14322, (2010).

35 McInnes, S. J. *et al.* Surface engineering of porous silicon to optimise therapeutic antibody loading and release. *Journal of Materials Chemistry B.* **3** (20), 4123-4133, (2015).
36 Prestidge, C. A. *et al.* Loading and release of a model protein from porous silicon powders. *physica status solidi (a).* **204** (10), 3361-3366, (2007).
37 Tzur-Balter, A., Young, J. M., Bonanno-Young, L. M. & Segal, E. Mathematical modeling of drug release from nanostructured porous Si: combining carrier erosion and hindered drug diffusion for predicting release kinetics. *Acta biomaterialia.* **9** (9), 8346-8353, (2013).
38 Nieto, A. *et al.* Surface Engineering of Porous Silicon Microparticles for Intravitreal Sustained Delivery of Rapamycin. *Investigative ophthalmology & visual science.* **56** (2), 1070-1080, (2015).
39 Warther, D. *et al.* Porous silicon based intravitreal platform for dual-drug loading and controlled release towards synergistic therapy. *Drug Delivery.* **25** (1), 1537-1545, (2018).
40 Li, W. *et al.* Tailoring Porous Silicon for Biomedical Applications: From Drug Delivery to Cancer Immunotherapy. *Advanced Materials.* **30** (24), 1703740, (2018).
41 Yazdi, I. K. *et al.* Physicochemical properties affect the synthesis, controlled delivery, degradation and pharmacokinetics of inorganic nanoporous materials. *Nanomedicine.* **10** (19), 3057-3075, (2015).
42 Fry, N. L., Boss, G. R. & Sailor, M. J. Oxidation-Induced Trapping of Drugs in Porous Silicon Microparticles. *Chemistry of Materials.* **26** (8), 2758-2764, (2014).
43 Jarvis, K. L., Barnes, T. J. & Prestidge, C. A. Surface chemistry of porous silicon and implications for drug encapsulation and delivery applications. *Advances in Colloid and Interface Science.* **175** 25-38, (2012).
44 Salonen, J. & Mäkilä, E. Thermally Carbonized Porous Silicon and Its Recent Applications. *Advanced Materials.* **30** (24), (2018).
45 Wang, M. *et al.* Influence of Surface Chemistry on the Release of an Antibacterial Drug from Nanostructured Porous Silicon. *Langmuir.* **31** (22), 6179-6185, (2015).
46 Salonen, J. *et al.* Mesoporous silicon microparticles for oral drug delivery: loading and release of five model drugs. *Journal of Controlled Release.* **108** (2), 362-374, (2005).
47 Park, J.-H. *et al.* Biodegradable luminescent porous silicon nanoparticles for in vivo applications. *Nature materials.* **8** (4), 331-336, (2009).
48 Johnson, P. J., Skornia, S. L., Stabenfeldt, S. E. & Willits, R. K. Maintaining bioactivity of NGF for controlled release from PLGA using PEG. *Journal of Biomedical Materials Research Part A.* **86** (2), 420-427, (2008).
49 Shefi, O. *et al.* Microtargeted gene silencing and ectopic expression in live embryos using biolistic delivery with a pneumatic capillary gun. *Journal of Neuroscience.* **26** (23), 6119-6123, (2006).
50 Zilony, N., Tzur-Balter, A., Segal, E. & Shefi, O. Bombarding Cancer: Biolistic Delivery of therapeutics using Porous Si Carriers. *Scientific Reports.* **3** 2499, (2013).
51 Zuidema, J. M., Provenza, C., Caliendo, T., Dutz, S. & Gilbert, R. J. Magnetic NGF-Releasing PLLA/Iron Oxide Nanoparticles Direct Extending Neurites and Preferentially Guide Neurites along Aligned Electrospun Microfibers. *ACS chemical neuroscience.* **6** (11), 1781-1788, (2015).
52 Baranes, K., Moshe, H., Alon, N., Schwartz, S. & Shefi, O. Neuronal Growth on l- and d-Cysteine Self-Assembled Monolayers Reveals Neuronal Chiral Sensitivity. *ACS chemical*

neuroscience. **5** (5), 370-376, (2014).
53 Marcus, M. *et al.* Interactions of Neurons with Physical Environments. *Advanced Healthcare Materials.* **6** (15), 1700267, (2017).