

SUPPLEMENTARY ONLINE MATERIAL

MATERIALS AND METHODS

Cell Culture and *In Vitro* Encapsulation in IKVAV-PA nano-networks

Neural progenitor cells (NPCs) were cultured as previously described (1). Briefly, the cortices of E13 mouse embryos were dissected and plated on un-treated petri dishes in DMEM/F12 media supplemented with bFGF (10 ng/ml). After four days, mechanically and enzymatically dissociated NPCs and undissociated neurospheres (i.e. undissociated NPC aggregates) were plated onto appropriate substrates (e.g. encapsulated in IKVAV-PA, EQS-PA, or alginate gels, or cultured on laminin, poly-D-lysine, or IKVAV peptide coated cover slips). In all cases this was taken as 0 days *in vitro*. Encapsulation of NPC in IKVAV- and EQS-PA networks was achieved by first aliquoting 100 μ l of PA solution onto a 12 mm cover slip in a 24 well culture plate, forming a self-contained drop. 100 μ l of cell suspension in culture media was then pipetted into the drop of PA solution, gently swirling the pipette tip as the cell suspension was being introduced, forming PA gels. Gels were allowed to sit undisturbed in the incubator (at 37°C and 5% CO₂, with 95% humidity) for > 2 hrs., after which 300 μ l of NPC culture media was added to the wells, completely submerging the PA gels. Plates were then returned to the incubator. Control 12 mm cover slips were coated with PDL (Sigma, 1 mg/50 ml DMEM) or laminin/PDL (Sigma, 1 mg/100 ml DMEM) and left to sit and dry in a flow hood for > 1 hour. Soluble IKVAV peptide was spin coated onto cover slips and allowed to dry overnight. For all the two dimensional controls, 300 μ l of NPC culture media was added to the wells, and 100 μ l of NPC cell suspension was aliquoted onto the center of the cover slip, followed by manual shaking of the culture plates to ensure a well distributed cell density. Alginate solutions at 1 wt% were made by mixing 1 g of alginate in 100 ml of physiological buffered saline (PBS) and left on a shaker overnight to allow it to dissolve. 100 μ l of 1 wt% alginate was mixed with 100 μ l of NPC cell suspension in culture media containing no exogenous calcium (which is normally required to induce alginate gelation), yielding 0.5 wt% alginate gels that would

allow direct comparisons with the 0.5 wt% IKVAV-PA experimental gels. The encapsulated NPC in the alginate were returned to the incubator for > 2 hours, by which time they had formed weak but stable gels. The culture wells were then filled with 300 μ l of culture media, enough to submerge the alginate gels, and returned to the incubator.

Cell Viability/Cytotoxicity Assay

Cell viability/cytotoxicity was assessed by using Molecular Probes LIVE/DEAD cell assay (Molecular Probes). Working concentrations of ethidium homodimer-1 (EthD) and calcein optimized for NPCs were determined as instructed by Molecular Probes, and were determined to be 0.5 and 8 μ M, respectively. The culture media was removed from the wells and enough EthD/calcein solution in PBS added to the wells to ensure submersion of the PA gels. Culture plates were returned to the incubator for 20 minutes, and then the EthD/calcein solution removed and the cells washed once with PBS. EthD and calcein fluorescence were imaged using FITC and TRITC filters, respectively, on a Nikon TE-2000 fluorescence microscope.

Immunocytochemistry

The culture media from encapsulated NPCs was removed and the encapsulated cells fixed with 4% paraformaldehyde for 20 minutes at room temperature by submerging the entire PA-gel in fixative. Incubation for 5 minutes with 0.2% Triton-X (t-octylphenoxypolyethoxyethanol) was preceded by two washes with PBS. This was followed by another two PBS washes and primary antibody incubation in PBS (anti- β -tubulin III IgG at 1:400 or anti-GFAP at 1:400, Sigma) containing 5% goat or horse serum overnight at 4°C. Following three washes with PBS the cells were incubated with TRITC- or FITC-conjugated secondary antibodies in PBS containing 5% goat or horse serum at room temperature for two hours. Following another three washes with PBS all nuclei were stained with Hoescht's Stain (1:5000, Sigma) for ten minutes at room temperature in order to visualize β -tubulin and GFAP negative cells. Cell imaging was done with a high resolution Cool Snap camera attached to a Nikon TE-2000 fluorescence microscope interfaced with a PC running MetaView imaging software, or an Axiocam

camera attached to a Zeiss Axiovert 200 fluorescence microscope interfaced with a PC running AxioVision imaging software.

Cell Counts

Randomly selected fields of view were imaged for different experimental conditions and cells counted using ImageJ (Scion Corporation) morphometric analysis software. Images were checked to make sure there was no bleed-through of fluorescence between filters, and cells semi-automatically counted using ImageJ. Specifically, the total numbers of cells within a given field were counted by manually selecting cells using a marking tool which kept an automatic running count of the total number of cells. Quantitative and statistical analyses of cell counts were done using Matlab (Mathworks) and/or Excel (Microsoft).

Spinal Cord Injection Procedure:

Rats were anesthetized using 45mg/kg Pentobarbital (Nembutal®). A laminectomy was performed to expose spinal segment T13 and a stereotaxic micromanipulator (Kopf Instruments) with a Hamilton syringe attached to a 32 gauge needle was used to inject 6µl at 333nl/sec of isoosmotic glucose (vehicle) or peptide amphiphile into the spinal cord at T10 at a depth of 1.5 mm. The needle was kept inside the site of injection for 2 minutes after each injection in order to allow the IKVAV-PA to gel without disturbance. Animals injected with peptide amphiphile showed no changes in locomotor behavior or general health, indicating that injection of the peptide amphiphile had no toxic effects.

Intra-ocular Injections

All experiments were done in accordance with the regulations of the Association for Research in Vision and Ophthalmology (ARVO) and Animal Care and Use Committee (ACUC) of Northwestern University. Adult Sprague-Dawley rats (200-250 g) were sacrificed by an overdose of Sodium Pentobarbital or CO₂ overdose and their

eyes immediately surgically enucleated. A 100 µl Hamilton syringe with a 25 G needle was pre-loaded with 80-100 µl of IKVAV-PA solution, and the enucleated eyes placed on the platform of a Nikon SMZ-1000 stereo dissecting microscope. The eyes were manually injected with IKVAV-PA solution into the back of the orbit under the stereo microscope at an oblique angle roughly into the sub-retinal or vitreal spaces, and imaged using the stereo microscope interfaced with a Cool Snap high resolution camera using MetaView imaging software.

Calculation of IKVAV Signal Amplification

The adsorption of proteins at a solid-liquid interface is typically in the vicinity of 1µg/cm² (2). Using this value, and given that the molecular weight of laminin is 800kDa (3), we can calculate that on a two-dimensional surface, such as a glass cover slip or a culture plate, the density of IKVAV epitopes on the surface is

$$\frac{10^{-6} g}{1 cm^2} \times \frac{mol}{800,000 g} \times \frac{6.023 \times 10^{23} molecules}{mol} = 7.53 \times 10^{11} molecules / cm^2$$

given that the number of IKVAV epitopes on a native laminin-1 molecule is one.

The density of IKVAV epitopes per square centimeter of a nanofiber surface can also be calculated using known fiber dimensions and molecular modeling. Given that the diameter of a single nanofiber is 7 nm, its circumference is 18.8 nm (C=2πd). Estimating from molecular dimensions that the fiber consists radially of 50 PA molecules, and that 1 cm = 10⁷ nm, we find that

$$10^7 nm \times \frac{50 PA molecules}{18.8 nm} = 2.7 \times 10^7 PA molecules / cm = 2.7 \times 10^7 IKVAV / cm$$

Assuming that the molecules, being otherwise unconstrained, will not preferentially elongate along one dimension or the other, we can square this to find the number of IKVAV epitopes per square centimeter of nanofiber surface as

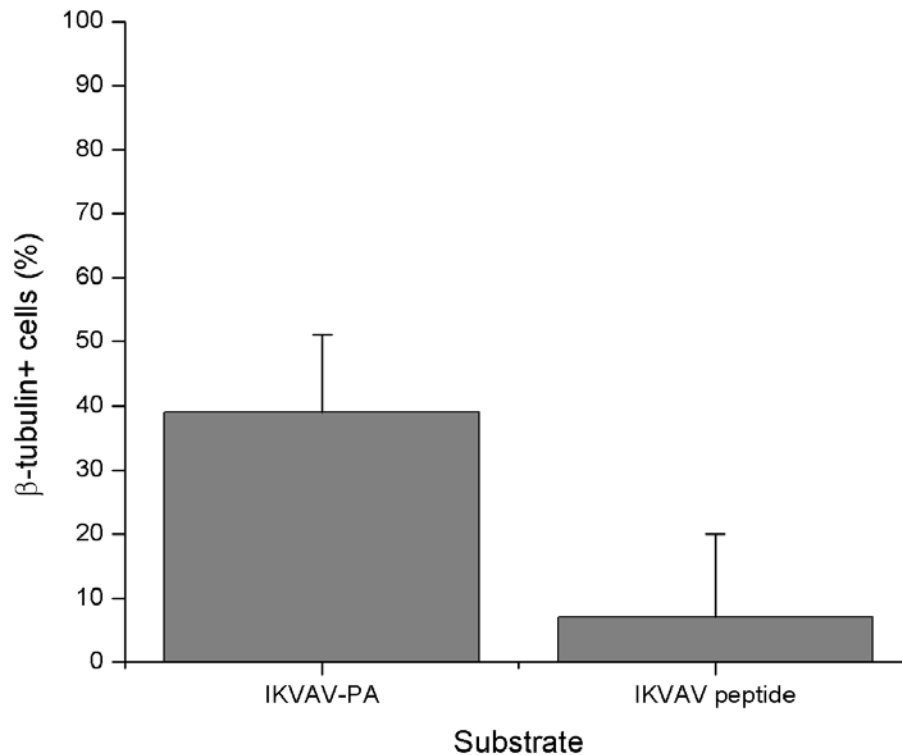
$$(2.7 \times 10^7 IKVAV / cm)^2 = 7.1 \times 10^{14} IKVAV / cm^2$$

We now divide these two numbers to find the ratio of IKVAV epitopes on a nanofiber to that on a two-dimensional surface, yielding the amplification factor of IKVAV epitopes on a nanofiber relative to a two-dimensional surface of closely packed laminin molecules:

$$\frac{7.1 \times 10^{14} IKVAV(PA) / cm^2}{7.53 \times 10^{11} IKVAV(lam) / cm^2} \approx 10^3$$

Two-dimensional cultures

For IKVAV peptide experiments, the same 12mm glass coverslips used for the three-dimensional experiments were soaked in ethanol to encourage hydrophilicity, then spin-coated with 50 μ L of a 1mg/mL IKVAV peptide solution. For IKVAV-PA experiments, the coverslips were coated with PDL (again, to encourage adsorption) and subsequently with IKVAV-PA solution. In both cases, the cover slips were allowed to dry overnight and then washed three times with distilled water to remove weakly adherent material before the addition of cell suspension. The results of β -tubulin staining after 1 DIV are shown in Supplementary Fig. 1.



Supplementary Fig 1. Percentage of total cells that differentiated into neurons in a two-dimensional culture on substrates coated with IKVAV-PA nanofibers and substrates coated with IKVAV peptide.

REFERENCES AND NOTES

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2. B. D. Ratner, *Biomaterials Science : An introduction to materials in medicine* (Academic Press, San Diego, 1996).
3. P. Tungal, N. Smyth, M. Paulsson, M. C. Ott, *Microsc. Res. Tech.* **51**, 214 (2000).