Supporting Information

Comparative analysis of aligned and random amniotic membrane-derived cryogels for neural tissue repair

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1. Materials and Methods

1.1. Cryogels fabrication

AMMA was synthesized following a modified version of the previously described method.[1] Human AMs were obtained from donated placentas under the agreement established between the University of Aveiro and the Centro Hospitalar do Baixo Vouga. Donors provided informed consent for the use of their placental tissue for research purposes. First, AMs underwent decellularization (dAM) with 1% (w/v) sodium dodecyl sulfate (SDS, Sigma-Aldrich, USA) solution over-night (with constant stirring at room temperature), followed by a 30 min treatment with 1% (w/v) Triton X-100 to eliminate cellular components. Upon cell lysis, the tissue was incubated in a nuclease solution, containing DNase (STEMCELL Technologies, Canada) and RNase (Thermo Fisher Scientific, USA), at 37ºC to further assist in the removal of nucleic acids. The resulting acellular matrix was enzymatically digested using pepsin (pH 2) under constant stirring for 72h, resulting in a solubilized matrix. The solution was freeze-dried and stored at 4 °C until further processing. To produce AMMA, lyophilized dAM was reacted with methacrylic anhydride (MA, Sigma-Aldrich) at room temperature for 4 hours. Residual MA was removed through dialysis against distilled water using SnakeSkin dialysis tubing with a 3.5 kDa molecular weight cut-off (Thermo Scientific) for 24h at room temperature.

The cryogel precursor solution was prepared by dissolving AMMA overnight at a concentration of 0.5 % (w/v) in a PBS solution containing 0.2 % (w/v) lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP, Sigma-Aldrich). To prepare anisotropic cryogels, 50 µL of the precursor solution was pipetted into moulds composed of an aluminium base and 6 mm diameter PDMS sides surrounded and covered by polystyrene foam. The moulds were put in contact with the freeze-dryer shelf at -80 °C for 1 hour. To prepare isotropic cryogels, the same volume of solution was pipetted into PDMS moulds and frozen inside petri dishes. The frozen gels were then removed from the freeze-dryer and immediately photopolymerized for 60 s using a multi-peak LED light curing unit (Valo™ Grand, Ultradent, USA). After curing and thawing, the cryogels were stored in PBS at 4 °C until further use.

1.2. Cryogels characterization

The morphology of freeze-dried cryogels was analysed from images acquired by scanning electron microscopy (SEM, Hitachi TM4000 plus, Japan) at an accelerating voltage of 15 kV. Pore size was measured using Fiji Image J software.

The rheological characterization of the cryogels' was conducted using a Kinexus Lab+ rotational rheometer (Malvern PANalytical, U.K.) equipped with an 8 mm diameter stainless-steel parallel plate geometry and a solvent trap. For shear modulus assessment, single frequency tests were performed at a constant shear strain of 1 % over a 5-minute period, with a sampling interval of 10 seconds. To explore potential variations in the response of anisotropic scaffolds to shear stress applied in different planes, rectangular cryogels (5x5x3 mm) were fabricated to facilitate testing in both vertical and horizontal configurations. The gap setting was selected to be 70 % of the scaffold height for all experiments, which were conducted at a temperature of 37 °C and a frequency of 1.0 Hz. Triplicate analysis was performed for all conditions.

To assess the mechanical compliance and shape memory properties of the cryogels, compression tests using tweezers and injection tests were performed, and the recovery time was measured. To validate the injectability of the scaffolds, rectangular cryogels were loaded into a 2 mL syringe with 1 mL of PBS and subsequently injected through an 18G needle into petri dishes filled with PBS.

1.3. Cell culture studies

Neuroepithelial stem cells (NE-4C, ATCC CRL-2935) were maintained on poly-L-lysine (PLL, Sigma-Aldrich) coated T-flasks with Eagle's Minimum Essential Medium (EMEM, Sigma-Aldrich) supplemented with 10 % (v/v) Foetal Bovine Serum (FBS, Sigma-Aldrich), and 1 % (v/v) Penicillin-Streptomycin (P-S, Sigma-Aldrich) at 37 °C and 5 % CO_2. The culture medium was refreshed every 2 days.

Cell culture: Prior seeding, AMMA cryogels were washed with PBS and UV-sterilized for 30 minutes. Cells were detached using trypsin/EDTA (Sigma-Aldrich), resuspended in culture medium, and seeded at a concentration of 200 000 cells in 5 µL. After 1 hour of attachment at 37 °C under 5 % CO_2, the scaffolds were covered with complete medium. For proliferation experiments, cells were cultured for up to 7 days. For differentiation experiments, cells were primed on day 4 with 10^{-6} M all-trans retinoic acid (RA, Sigma-Aldrich) in a low serum medium (EMEM supplemented with 1 % FBS and 1 % P-S) for 2 days. Post-priming, cells were cultured for an additional 8 days in low serum medium, with the medium changed every other day.

Cell viability analysis: Viability assessment using the Live/Dead assay was conducted on days 1, 4, and 7. The samples were incubated in a culture medium containing 1:2000 of 1 mg/mL Calcein AM (Invitrogen) and 1:500 of 2 mM Ethidium homodimer (Sigma-Aldrich) for 30 minutes. Subsequently, the cryogels were observed using a confocal microscope (ZEISS LSM 900, Carl Zeiss, Germany).

Cell Metabolic Activity Quantification: The metabolic activity of NSC was assessed using the resazurin reduction assay on days 1, 4, and 7. A solution of resazurin (0.1 mg/mL; ACROS Organics) in PBS was introduced into the culture medium, resulting in a final concentration of 10 % (v/v). After 4 hours of incubation at 37 °C, triplicates of 50 µL per sample were transferred to a 96-well plate, and the absorbance was measured at 570 and 600 nm wavelengths. Absorbance values were determined by subtracting the absorbance at 600 nm from that at 570 nm, with subsequent subtraction of the mean values of the control well.

Cell proliferation by DNA quantification: At pre-determined time points, cell culture medium was removed from the wells and the cryogels washed with PBS and frozen at -80 °C until further use. Prior DNA quantification, cryogels were thawed and digested in 500 µL of 0.1 M Sodium phosphate dibasic (Sigma-Aldrich), 10 mM Ethylenediaminetetraacetic acid disodium salt dihydrate (Sigma-Aldrich), 10 mM L-Cysteine hydrochloride (Sigma-Aldrich) and 3.8 U/mL Papain (Roche) in deionized water for 16 hours at 60 °C. The digest was diluted in 1X TNE buffer to a final volume of 1 mL. After that, 1 mL of 2X Dye Solution (Hoechst 33258, 200 ng/mL, Sigma-Aldrich) was added to the previous mixture and triplicates of 200 µL per sample were transferred to a 96-well plate. DNA standards were prepared from Calf Thymus DNA (Sigma-Aldrich) with concentration ranging from 0 to 1 µg/mL. The fluorescence was measured using an excitation wavelength of 348 nm and an emission wavelength of 456 nm (Synergy HTX, BioTek).

Cell morphology analysis: To investigate NE-4C morphology and migration, cells were stained with phalloidin and DAPI. On day 7, cells were fixed with a 4 % (w/v) Paraformaldehyde (PFA, Sigma-Aldrich) solution in PBS for 15 minutes, followed by treatment with 0.1 % (v/v) Triton X-

100 (Fisher Scientific) in PBS for 10 minutes. To block nonspecific binding, samples were incubated with 5 % (v/v) FBS in PBS for 30 minutes. The samples were then incubated with Flash Phalloidin Red 594 (1:40, BioLegend) for 45 min at room temperature. Subsequently, cell nuclei were stained with DAPI (Sigma-Aldrich) at a concentration of 0.1 μg/mL in PBS for 10 minutes. Between each step and after the final step, the samples were washed with PBS. Finally, the samples were mounted on glass microscope slides and analysed under the confocal microscope.

Cell Differentiation analysis: An immunolabeling procedure was used to investigate NE-4C differentiation. Samples were fixed with 1X Fixation Buffer (F1797, Sigma) for 15 minutes at room temperature. Subsequently, they were permeabilized with Triton X-100 as described above and blocked with 2 % (v/v) Bovine Serum Albumin (BSA, Sigma-Aldrich). For labelling neurons and astrocytes, cells were incubated with the primary antibodies mouse anti-β-tubulin 3 (1:500, Biolegend), also known as TUJ-1, and rabbit anti-glial fibrillary acidic protein (GFAP, 1:250, Sigma-Aldrich), respectively, for 1 hour at room temperature. The samples were then incubated with the secondary antibodies Goat anti-Mouse IgG (H+L) DyLight-488 (1:500, Invitrogen) and Goat anti-Chicken IgY (H+L) Alexa Fluor-594 (1:500, Invitrogen), again for 1 hour at room temperature. For labelling synapses, Anti-Synaptophysin antibody (1:100, Abcam) and Goat anti-Rabbit IgG (H+L) Alexa Fluor-594 (1:500, Invitrogen) were used as primary and secondary antibodies, respectively. To label cell nuclei, DAPI at a concentration of 0.1 μg/mL in PBS was added for 10 minutes. After each step, samples were washed three times with PBS. Finally, samples were mounted onto glass microscope slides and examined using the confocal microscope. The area positively stained for each particular marker was calculated using Fiji Image J software.

1.4. Statistical analysis

Statistical analysis was carried out using Origin software, presenting the outcomes as mean ± standard deviation. Statistical significance was assessed through one-way ANOVA analysis paired with Tukey's multiple comparisons test (*$p < 0.05$; **$p < 0.01$; and ***$p < 0.001$).

2. Supplementary Figures

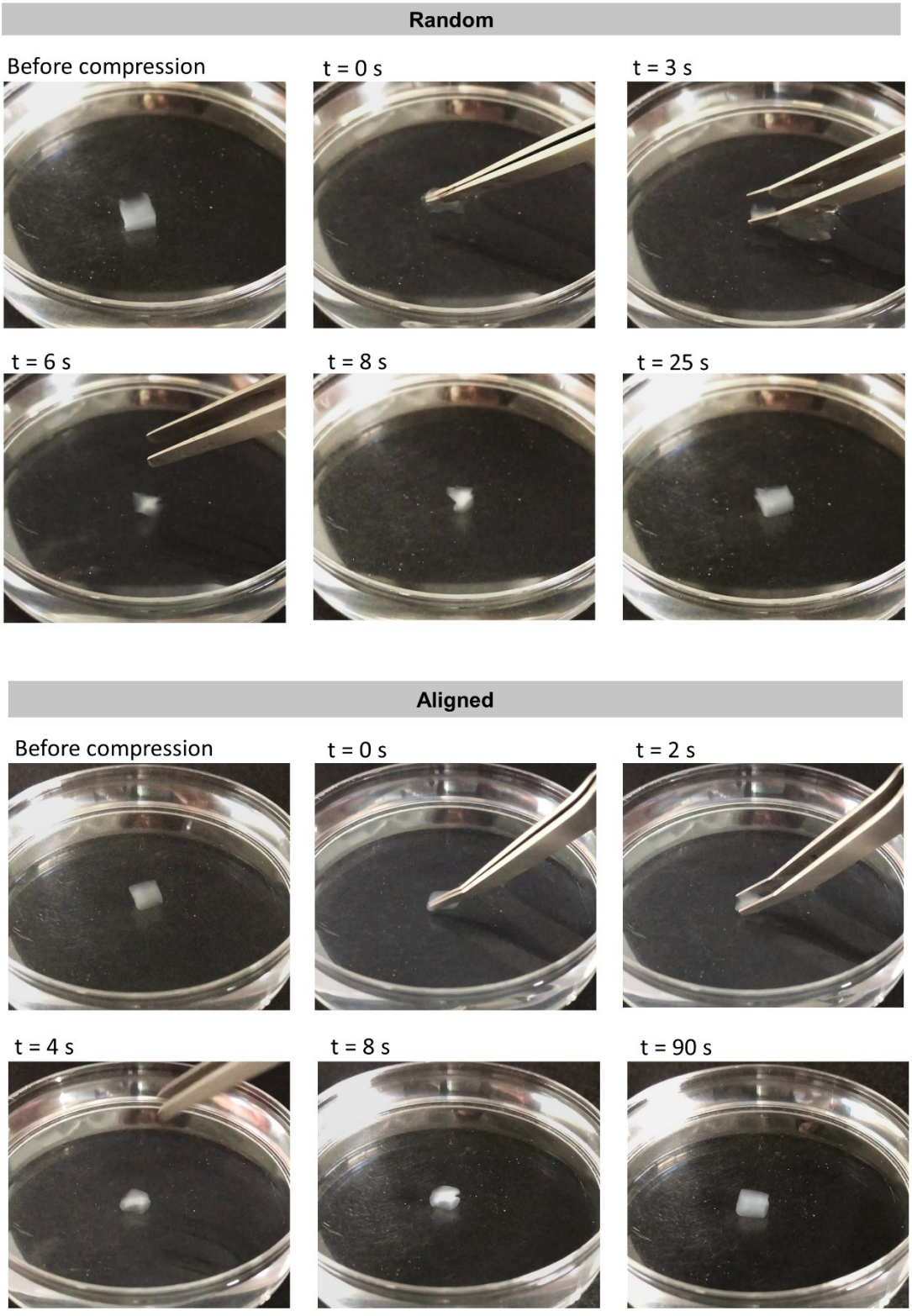

Figure S1 – Shape recovery capabilities and corresponding recovery times of random and aligned cryogels compressed with tweezers and immersed in PBS.

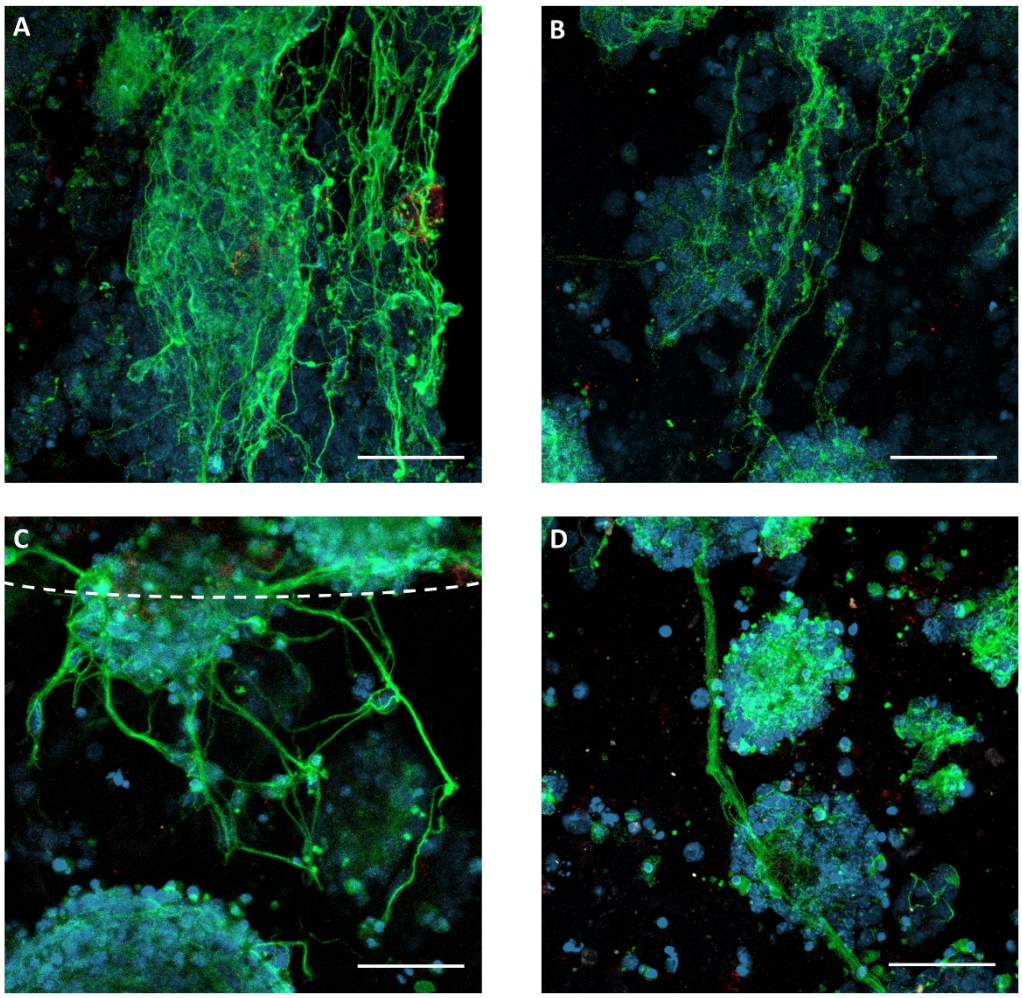

Figure S2 – Images of the cross-sections of aligned cryogels illustrating the extension of neurites 10 days after differentiation induction with RA. Neurons are labelled for TUJ-1 (green), astrocytes for GFAP (red), and cell nuclei with DAPI (blue). Scale bars = 50 µm.

References

(1) Deus, I. A.; Santos, S. C.; Custódio, C. A.; Mano, J. F. Designing Highly Customizable Human Based Platforms for Cell Culture Using Proteins from the Amniotic Membrane. *Biomater. Adv.* **2022**, *134*, 112574. https://doi.org/10.1016/j.msec.2021.112574.