Supplementary Materials

Flexible and immunomodulatory milk-derived protein hydrogels as transient interfacing scaffolds for peripheral nerve integration

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Supplementary Experimental Section/Methods

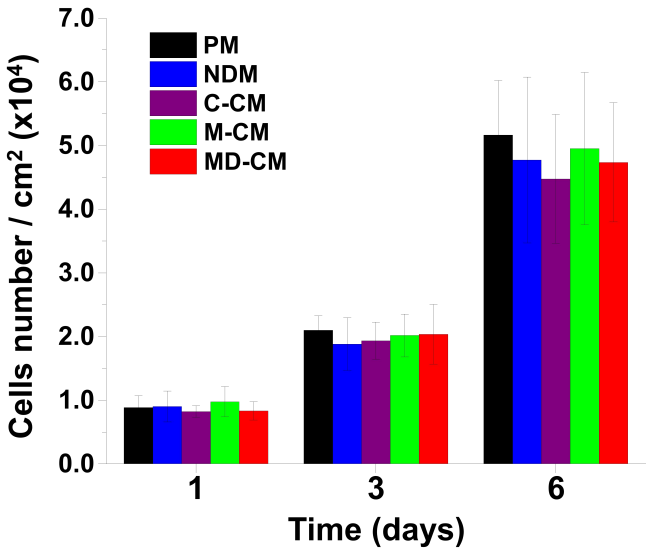
PC12 cell density and proliferation quantification: To evaluate the potential cytotoxicity and cell-handling cytocompatibility of the bioactive molecules naturally liberated from the hydrogel matrices, PC12 cells were seeded at an initial density of 5 x 10^3 cells/well onto 96-well culture plates (Corning Inc., Corning, NY, USA). Rather than using artificial enzymatic digests, the cells were maintained under five distinct microenvironments to track the biosafety of spontaneous matrix leaching (proliferation media (PM), neuronal differentiation media (NDM), and macrophage-conditioned media derived from TCPS (C-CM), MDP (M-CM), and MDP-DOPA (MD-CM) groups. The volumetric mixing ratio of each collected conditioned medium to the base differentiation medium was strictly maintained at 6.5:3.5 (v/v) to establish synchronized sub-optimal screening backgrounds. At localized incubation intervals of 1, 3, and 6 days, the absolute number of viable PC12 cells was quantified by digital image-based profiling. Briefly, random high-magnification phase-contrast optical micrographs were systematically captured across multiple predetermined fields of view per culture matrix replicate. The values were normalized to the unit surface area to yield the final cell density (ells/cm^2 x 10^4).

Cytokine protein array profiling: To characterize the functional secretory profile of macrophages at the protein level, the culture supernatants of LPS-activated RAW 264.7 cells cultivated on either MDP or MDP-DOPA hydrogels for 24 hours were harvested. The collected supernatants were centrifuged at 300 g for 20 minutes and filtered through a 0.45 μm syringe filter to remove cellular debris. A multiplexed membrane-based cytokine antibody array kit (Proteome Profiler Mouse Cytokine Array XL, Catalog Number: ARY028, R&D Systems, Minneapolis, MN, USA) targeting distinct inflammatory cytokines, chemokines, and growth factors was utilized according to the manufacturer's instructions. Chemiluminescent signals captured from the array membranes were digitized and quantified via image J. The pixel intensities of individual cytokine spots were normalized to the internal positive controls on each membrane, and the relative protein expression levels were visualized as a hierarchical clustering heatmap.

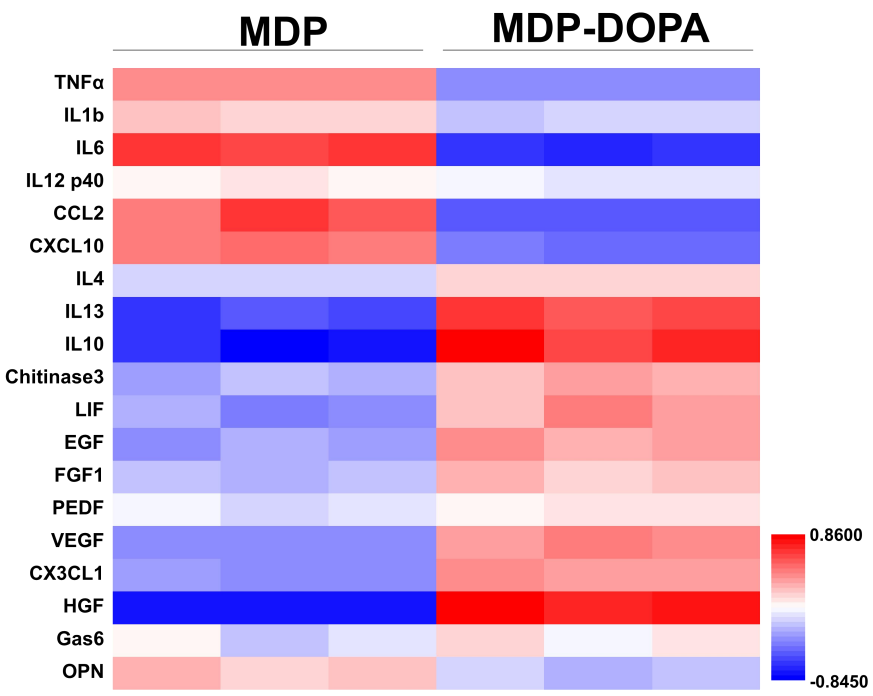
Cumulative protein release kinetics : To evaluate the release profiles of bioactive milk-derived protein components from the hydrogels, the cumulative protein release was

monitored over 28 days. Disc-type hydrogel substrates measuring 10 mm in diameter were prepared for both MDP and MDP-DOPA groups. Each substrate was immersed in 1 mL of phosphate-buffered saline (PBS, pH 7.4) and incubated at a physiological temperature of 37 °C under continuous shaking at 120 rpm. At predetermined incubation intervals of 1, 3, 5, 7, 14, and 28 days, the total volume of the supernatant was completely harvested and replaced with an equal volume of fresh PBS to maintain sink conditions. The concentration of the liberated proteins in the retrieved supernatants was quantified using a bicinchoninic acid (BCA) protein assay kit (Catalog Number, Manufacturer, City, State, Country) according to the manufacturer's protocol. The absorbance was measured at 562 nm using a multimode microplate reader (Spark 20M, Tecan, Männedorf, Switzerland). The percentage of cumulative protein release was calculated relative to the initial total protein content loaded within the hydrogel matrices

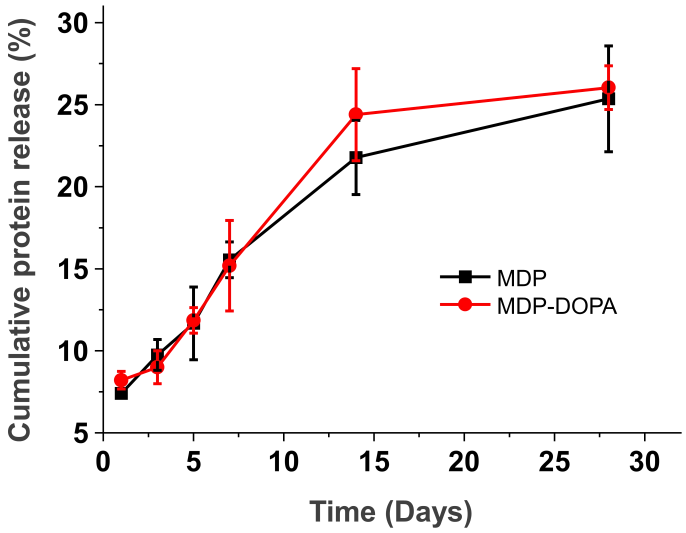
Semi quantitative reverse transcription polymerase chain reaction analysis: To verify the immunomodulatory effects on conventional control matrices, semi quantitative reverse transcription polymerase chain reaction (RT-PCR) was performed using RAW 264.7 cells cultured on polycaprolactone (PCL) and poly(DOPA)-coated PCL (PCL-DOPA) films. Cells were seeded at a density of 2 x 10^5 cells/patch and stimulated with 100 ng/mL of lipopolysaccharide (LPS) for 24 hours. Total RNA was extracted using a Total RNA Isolation Kit (AccuPrep® Universal RNA Extraction Kit, K-3140, Bioneer, Daejeon, Republic of Korea) and reverse transcribed into complementary DNA (cDNA) using a cDNA reverse transcription kit according to the manufacturer's protocol. The target cDNA sequences for M1-associated genes (TNF-α, CD86, CCL19, CXCL11) and M2-associated genes (IL-10, CD206, CCL13, CD163) were amplified using specific primers and a PCR master mix. The amplified PCR products were separated by agarose gel electrophoresis and visualized under ultraviolet light. The relative intensities of the nucleic acid bands were quantified by densitometric analysis using ImageJ software. Target gene expression levels were normalized to the GAPDH housekeeping control band.



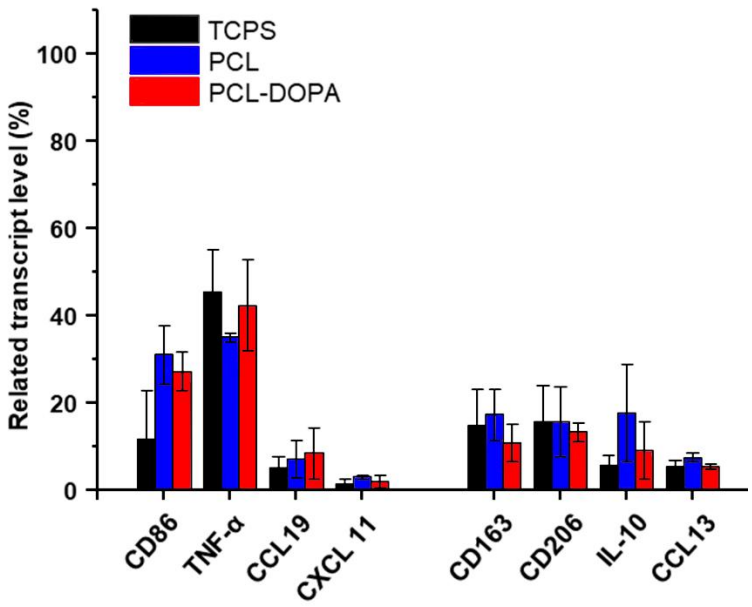
Supplementary Figure 1. Quantitative assessment of PC12 cell density and biosafety profiling of hydrogel extracts. Time-course quantification of total adhered PC12 cell density (cells/cm^2 x 10^4) monitored over a cultivation period of 1, 3, and 6 days under various experimental microenvironments. The stable cell proliferation behavior observed across all groups confirms the cytocompatibility and absence of cytotoxicity of the MDP-DOPA hydrogel matrix and its spontaneously liberated extract components. Quantitative data and corresponding error bars are expressed as the mean ± standard deviation (mean ± SD) calculated from three independent experiments using multiple biological replicates (n = 3 per group at each time point).



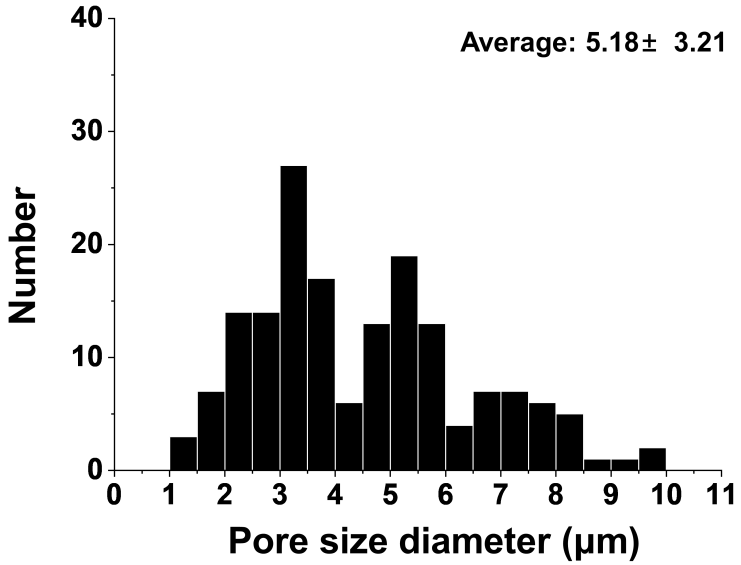
Supplementary Figure 2. The hierarchical clustering heatmap illustrates the relative expression profiles of 19 distinct inflammatory cytokines, chemokines, and growth factors secreted by LPS-activated RAW 264.7 cells cultured on MDP or MDP-DOPA hydrogel substrates. The MDP-DOPA interface alters the functional macrophage secretory profile, suppressing pro-inflammatory M1 factors while elevating pro-healing and neurotrophic growth factors. The color scale denotes the normalized Z-score of relative protein expression levels ranging from red for high secretion to blue for low secretion.



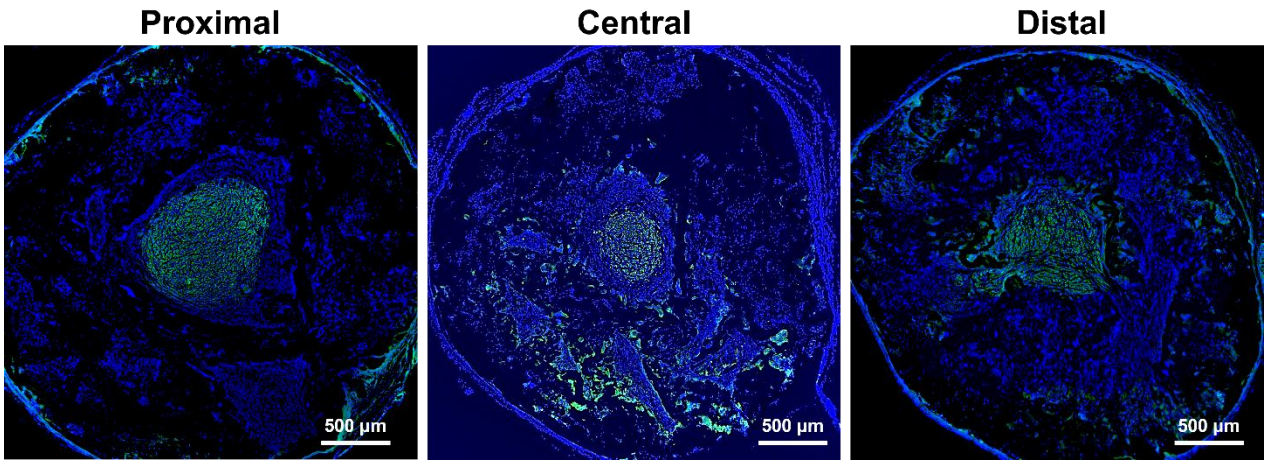
Supplementary Figure 3. In vitro cumulative protein release kinetics of hydrogel scaffolds. Cumulative milk-derived protein release profiles from MDP and MDP-DOPA hydrogel substrates monitored over a cultivation period of 28 days in PBS at 37 °C. Both groups exhibit a stable and sustained protein liberation behavior without a noticeable initial burst release. Quantitative data and corresponding error bars are expressed as the mean ± standard deviation (mean ± SD) calculated from three independent experiments using multiple biological replicates (n = 3 per group at each time point).



Supplementary Figure 4. Relative transcript levels of M1 associated genes (CD86, TNF-α, CCL19, CXCL11) and M2 associated genes (CD163, CD206, IL-10, CCL13) in LPS activated RAW 264.7 cells after 24 hours of cultivation on TCPS, PCL, or PCL-DOPA films. The relative gene expression levels were determined by densitometric quantification of the amplified PCR product bands separated via agarose gel electrophoresis. All transcript values were normalized to the corresponding GAPDH internal control band. Quantitative data and corresponding error bars are expressed as the mean ± standard deviation (mean ± SD) calculated from three independent experiments using multiple biological replicates (n = 3 per group).



Supplementary Figure 5. The histogram illustrates the size frequency distribution of internal pores quantified from cross sectional SEM micrographs of the hydrogel scaffold. The structural porosity was analyzed using ImageJ software by evaluating randomly selected pore diameters. The average pore diameter of the fabricated matrix is determined to be 5.18 ± 3.21μm.



Supplementary Figure 6. Representative cross sectional fluorescence images of the regenerated nerve tissue harvested 8 weeks post implantation. The hydrogel conduit maintains its defined circular geometry and physical tubular structure without structural collapse or deformation in the in vivo environment. This robust mechanical integrity preserves an adequate luminal space to support and guide functional axonal regeneration. Blue denotes DAPI nuclei counterstaining and green represents the regenerated neural components.