

Advanced microfluidic strategy for In-Bead MSC spheroid formation and co-encapsulation of necrosis inhibitor-loaded nanoparticles

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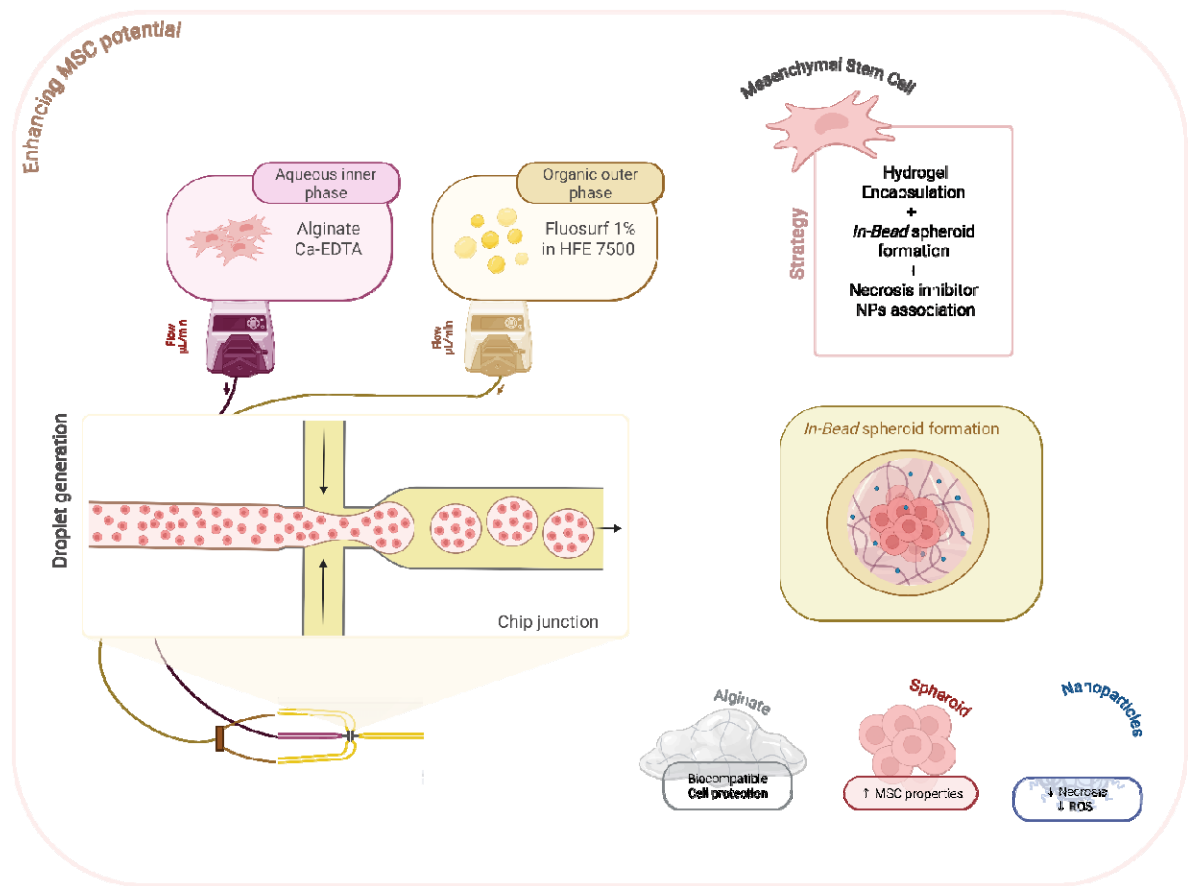
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Abstract: Mesenchymal stem/stromal cells (MSCs) are key players in regenerative medicine due to their immunomodulatory properties and ability to promote tissue repair. However, their therapeutic efficacy is often limited by rapid clearance following transplantation. MSC spheroids have shown enhanced functional properties, and we hypothesize that encapsulating them within hydrogel microbeads could offer additional protection and improve their viability. In this study, we developed a novel droplet-based microfluidic protocol for human MSCs derived from the apical papilla (SCAP) encapsulation and *In-Bead* spheroid formation within alginate microbeads. Optimization of the protocol allowed the formation of MSC spheroids in alginate droplets overnight (*In-Bead*), before alginate cross-linking and retrieval of alginate beads loaded with MSC spheroids. SCAP were successfully encapsulated within 275 μm alginate microbeads, forming spheroids of approximately 80 μm in diameter. Encapsulated SCAP spheroids retained their immunomodulatory properties. The process was further optimized by incorporating nanomedicines into the alginate solution before the formation of droplets and then spheroids, forming thus hybrid beads (Sph.Beads/NP). Nanomedicines were loaded with NecroX-5, a necrosis inhibitor, to improve SCAP viability further. Live/Dead assays indicated a protective effect of the nanomedicines, supporting the potential of this system for advanced cell delivery in regenerative applications.

Keywords: mesenchymal stem/stromal cell; microfluidics; alginate; spheroids; Necrox-5; micro beads; dental stem cells; nanomedicines; cell viability



Graphical abstract

A combination strategy enhancing MSC viability through spheroid formation, microencapsulation, and nanomedicine association achieved by microfluidic encapsulation with *In-Bead* spheroid formation. Created with BioRender

I. INTRODUCTION

Regenerative medicine seeks to repair and restore the function of damaged tissues or organs [1]. Mesenchymal stem/stromal cells (MSCs) play a central role in regenerative strategies, both in tissue engineering and cell therapy, due to their ability to support tissue regeneration [2] and modulate the local microenvironment through their diverse secretome [3]. Among alternatives to bone marrow-derived MSCs, dental stem cells, particularly stem cells from the human apical papilla (SCAP), have garnered growing attention [4]. SCAP are especially promising for neuroregeneration, as they originate from the neural crest [5] and have been studied for their potential in spinal cord injury repair [6, 7].

However, the therapeutic efficacy of MSCs remains limited, primarily due to poor survival and low retention following transplantation. Whether administered locally or systematically, MSCs often disperse throughout the body, resulting in low accumulation and retention at the target site. Additionally, the hostile environment, combined with potential nutrient deficiencies [8] and immune rejection [9], further compromises their viability. These challenges lead to significant cell death through apoptosis and necrosis shortly after transplantation, substantially reducing the overall therapeutic potential of MSCs [10].

A variety of strategies have been developed to improve MSC survival after transplantation. These include preconditioning techniques (eg. hypoxic or cytokine priming), genetic modifications, and encapsulation in scaffolds [11]. While MSC spheroid formation, hydrogel encapsulation, and nanomedicine-based exogenous support have each been explored as independent strategies to enhance cell survival and function, their synergistic integration represents a novel and particularly promising avenue. By combining these approaches, it may be possible to leverage the enhanced immunomodulation and stemness of spheroids [12-14], the controlled microenvironment and immune protection of hydrogels [15, 16], and the

targeted, sustained delivery enabled by nanomedicines [17], thereby addressing multiple limitations of MSC transplantation in a unified system.

Several biomaterials, such as fibrin [18, 19], hyaluronic acid [20, 21], gelatin [22, 23], and collagen [24, 25], have proven effective protection as hydrogel matrices for MSC therapy. Among these, alginate stands out as one of the most widely studied materials, with decades of use for encapsulating and transplanting pancreatic islets for the treatment of Type 1 diabetes [26]. Alginate cross-links via interactions with divalent ions, primarily calcium (Ca^{2+}), with gelation triggered by CaCl_2 addition, pH-dependent CaCO_3 dissolution, or chelation with EDTA [27, 28]. Its cytocompatibility and tunable properties make alginate a preferred biomaterial for cell delivery applications [29]. When encapsulated in alginate hydrogels, MSCs demonstrate prolonged secretome release, with peak activity timing depending on the specific activation protocol [30].

Further enhancement of MSC properties and post-transplantation survival can be achieved through exogenous support, such as the co-administration of bioactive molecules. This includes growth factors (e.g., VEGF [31], BDNF [6]) or small molecules (e.g., silibinin [32], rapamycin [33]), typically delivered via hydrogel co-encapsulation or micro/nanocarrier association to protect them and enable their sustained release. Among the critical challenges to MSC survival, necrosis plays a major role, driven in part by oxidative stress. NecroX-5, a potent necrosis inhibitor, mitigates oxidative cell death by scavenging reactive oxygen species (ROS) [34, 35]. Building on this, we previously optimized encapsulation of NecroX-5 in polymeric nanoparticles (NPs), which provided prolonged necrosis inhibition, thereby improving cell viability [17, 36]. We therefore hypothesized that combining MSCs with NecroX-5-loaded nanomedicines, particularly within the protective microenvironment of alginate hydrogels, could synergistically enhance cell survival by addressing both oxidative damage and the hostile post-transplantation environment.

While we previously demonstrated the fabrication of MSC spheroids combined with NPs using a magnetic bioprinting technique [17], the association efficiency between cells and NPs remained suboptimal. To overcome this limitation and further enhance MSC viability, we explored cell microencapsulation as a dual strategy: not only does it address the well-documented benefits of immune protection and controlled microenvironment, but it also enables the co-encapsulation of MSCs and NPs in close spatial proximity, fostering their synergistic interaction. To achieve this, we employed droplet-based microfluidics, a technology that precisely manipulates fluids at the microscale, to generate alginate hydrogel beads containing both MSC spheroids and NPs [37]. Unlike conventional methods such as electrostatic extrusion, microfluidics offers unparalleled control over droplet formation, yielding hydrogel microbeads with uniform size, high throughput production, and tunable cross-linking dynamics. This level of precision ensures a consistent and reproducible microenvironment, critical for optimizing cell viability and function [38]. While droplet-based microfluidics is widely established for single-cell encapsulation, its application to spheroid-nanoparticle co-encapsulation remains underexplored, highlighting the novelty of our approach [39].

We therefore hypothesized that integrating MSC spheroids formed *in situ* with NecroX-5-loaded NPs within a single microfluidic-generated alginate microsystem would synergistically combine their respective advantages, yielding hybrid beads (Sph.Beads/NP) with enhanced MSC viability and therapeutic potential.

To test this, we developed a novel microfluidic protocol for generating MSC spheroids directly within alginate microbeads (*In-Bead*). Unlike conventional approaches, where spheroids are pre-formed before encapsulation, our method enables spheroid formation within pre-gel alginate droplets, followed by controlled cross-linking. This ensures optimal bead structural integrity while preserving spheroid viability, addressing a key limitation of existing

techniques. Additionally, we investigated the co-encapsulation of NecroX-5-loaded nanomedicines within these alginate beads and evaluated their impact on MSC survival and function.

By merging biomaterial-based cell engineering with targeted nanotherapeutics, this approach creates a supportive microenvironment that not only protects MSCs from oxidative stress and necrosis but also enhances their regenerative efficacy. This innovative strategy holds significant promise for improving MSC-based therapies in regenerative medicine.

II. MATERIALS & METHODS

II.1. Materials

Low viscosity alginic acid sodium salt extracted from seaweed was purchased from Sigma Aldrich (no. 180947, lot MKCS7881 with M/G ratio 1.56, molecular weight 12-80 kDa, 17 cP for 1% in H₂O) and further purified by charcoal treatment and filtration on 0.22 µm before freeze-drying and storage at -20°C. NecroX-5 (methanesulfate) and NecroX-2 were purchased from Enzo Life Sciences (New-York, NY, USA). Fluosurf 2% and HFE 7500 were purchased from Unchained Labs (Pleasanton, CA, USA). PFO (1H,1H,2H,2H-Perfluoro-1-octanol) was purchased from TCI Chemicals (Tokyo, Japan). Calcein-AM was purchased from Biotium (Fremont, CA, USA). Resomer 502H® (D, L-lactide-co-glycolide) (PLGA) was a kind gift of EVONIK (Essen, Germany). DiD' solid; DiIC18(5) solid (1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt), TRIzol® Reagent, FluoReporter™ Blue Fluorometric dsDNA Quantitation Kit, and Ethidium homodimer D-1 were purchased from inVitrogen, Thermo Fischer Scientific (Waltham, MA, USA). Penicillin/Streptomycin (PEST), HEPES (1M) buffer, and StemPro™ Accutase™ were purchased from Gibco™, Thermo Fischer Scientific (Waltham, MA, USA). GoScript Reverse transcriptase and GoTaq® qPCR Master Mix were purchased from Promega (Madison, WI, USA). Poly(ethylene glycol) methyl ether-block-poly(lactide-co-glycolide) (PEG-PLGA – PEG average Mn 5,000, PLGA Mn 7,000, ratio 0.7), Sodium Cholate hydrate, EDTA calcium, Minimum Essential Medium (MEM) Eagle alpha, Fetal Bovine Serum (FBS), Ammonium formate LiChropur™ LC/MS, HiPerSolv CHROMANORM Formic acid 99% LC/MS were purchased from Sigma Aldrich (St. Louis, MO, USA). Recombinant Human TNF-α, Recombinant murine TNF-α, Recombinant Human IFN-γ, and Recombinant murine IFN-γ were purchased from PEPROTECH (London, UK).

II.2. Microfluidic method for forming SCAP spheroids in alginate microbeads

II.2.1. Cell-loaded alginate droplet generation by flow-focusing microfluidics

Dental stem cells from human apical papilla (SCAP) (RP89 cells, Ruparel 2013 [40]) were obtained from and characterized by Prof. Diogenes (San Antonio, Texas). According to Belgian regulations, cells were registered in UCLouvain Biobank (BB190051). SCAP were cultured in alpha-MEM medium supplemented with 10% FBS and 1% PEST. The medium was changed every two days, and cells were passaged once a week. SCAP expressing Green Fluorescent Protein (GFP+) and luciferase (Luc+) were obtained by lentivirus transduction, kindly performed in the Michiels' lab (de Duve Inst., UCLouvain), as previously described [17, 41, 42].

A 0.5% (w/v) alginate solution was incubated with activated charcoal for 30 min under stirring, before filtration through a 0.22 μ m filtration unit. The filtered solution was aliquoted, freeze-dried, and stored at -20°C. A 4% (w/v) sodium alginate solution in Ultrapure™ water was prepared before bead production. For bead production, SCAP were retrieved (Accutase™) and resuspended at 5.10^6 cells/mL in alginate (0.75% w/v), Ca-EDTA (37.5 mM), and complete cell culture medium. Using a Dolomite™ microfluidics system (Unchained Lab) connected to a Fluorophilic chip (190 μ m), alginate droplets containing cells were produced using a continuous organic phase of HFE 7500 supplemented with Fluosurf® 1%. Flow rates were set at 20 μ L/min for the oil phase and 10 μ L/min for the alginate/cell phase. Magnetic stirring was applied to the alginate/cell mix (100rpm) to maintain the SCAP in suspension. Droplet generation and cell encapsulation were monitored using a high-speed microscope (Supplementary Video 1). Droplets were collected in 1.5 ml Eppendorf tubes over a 5-minute period per sample. The samples containing the droplets were further incubated at 37°C. Each 5-minute collection, corresponding to the introduction of 250,000 cells into the microfluidic system, was defined as a sample ($n=1$). Several successive

samples obtained on the same day from the same cell batch and alginate dilution constituted one production cycle, which was considered an experimental replicate ($N=1$).

II.2.2. Cross-linking of alginate microbeads containing SCAP spheroids (Sph.Beads)

Droplets were collected and incubated at 37°C for 18h to allow the formation of spheroids (*In-Bead* formation) [43]. Then, alginate microbeads containing SCAP spheroids (Sph.Beads) were cross-linked by the addition of acetic acid (0.1%). Beads were washed and stabilised using PFO (>98%) and cell medium containing 25 mM HEPES, 1% (w/v) Pluronic F127, and 40 mM CaCl_2 [28]. An anti-static gun was employed to facilitate phase separation when required. Sph.Beads were incubated at 37°C in cell medium (10% FBS) until further use. To produce non-encapsulated spheroids, droplets formed by microfluidics were collected and disrupted without alginate cross-linking to retrieve the spheroids.

II.3. Sph.Bead characterization

II.3.1. Bead morphology and cell content

Alginate droplets were imaged using an optical microscope EVOS XL core (Life Technologies) before and after cross-linking. Bead and spheroid sizes were measured using ImageJ ($N=5$, $n=10$). Non-encapsulated spheroids were used to assess the spheroid yield and the number of cells per spheroid. The spheroid yield, number of spheroids produced per sample, was determined post-production after counting (optical microscope acquisition of whole sample in a 24-well – merge of 40 images) ($N=2$, $n=3$). The number of cells per spheroid was quantified using the FluoReporter® Blue Fluorometric dsDNA Quantification kit according to the manufacturer's protocol. Spheroid samples and SCAP suspensions containing a known number of cells were collected, lysed by adding water, followed by incubation at 37°C, and subsequently stored at -80°C. Samples were thawed and vortexed to

retrieve DNA. DNA was further stained with Hoechst 33258. Fluorescence was measured (Ex/Em 360-460) using a SpectraMax ID5 (Molecular Devices) (N=3, n=3).

II.3.2. Sph.Bead cell viability by Live/Dead assay

Cell viability of Sph.Beads was assessed using a Live/Dead assay. After 1, 4, and 7 days in culture, a fraction of the beads was transferred to a new plate using a sterile plastic pipette Pasteur (~200 μ L, 1/5 of the sample, ~500 beads), and the cells were stained with Calcein-AM (1 μ M) and Ethidium Homodimer (4 μ M) for 1 hour. Samples were analyzed by fluorescence microscopy using an LSM-980 Multiphoton confocal (ZEISS) (N=2, n=3).

II.3.3. Sph.Bead expression of immunomodulatory molecules

The impact of spheroid encapsulation in alginate beads on SCAP gene expression of immunomodulatory molecules in response to a pro-inflammatory environment was evaluated using RT-qPCR.

Sph.Beads were placed in a 24-well plate, each well containing a sample (5-minute collection – 250,000 cells – N=2, n=3), and underwent progressive FBS depletion to achieve a final FBS concentration of 0.75%, through progressive medium changes with FBS-free medium (Pest 1%) over the course of 24 hours. Cells were then activated with human-TNF α (20 ng/mL) & IFN γ (20 ng/mL), using non-activated spheroids as controls [44]. Forty-eight hours post-activation, Sph.Beads and spheroids were retrieved and transferred to Lysis Matrix D tubes (MP Biomedicals, Irvine, CA, USA) containing Trizol (500 μ L). Both were frozen at -80°C for subsequent mRNA expression analysis. Gene expression of Sph.Beads determined as described in II.3.5, was compared to non-encapsulated SCAP spheroids produced as described in II.2.

II.3.4. Sph.Bead immunomodulatory impact on activated microglia

The impact of Sph.Beads on the gene expression of activated murine microglial cells, BV2 cells, was assessed by RT-qPCR. BV2 cells were seeded in a 24-well plate (50,000 cells/well) and were incubated with murine TNF α and IFN γ (20ng/mL) for 16h. Non-activated BV2 (M0) were used as controls of activation. Activated BV2 cells were incubated with Sph.Beads in SCAP medium supplemented with TNF α and IFN γ for 3 days. Empty alginate beads were used as negative controls. Each well was treated with the equivalent of 250,000 cells. Then, medium was removed, and 200 μ L of Trizol were added to the BV2 cells before storage at -80°C for subsequent mRNA expression analysis (N=3, n=4).

II.3.5. RNA extraction and real-time q-PCR

Total RNA was extracted from Sph.Beads and spheroids using the Precelys Evolution system (60s, 2x, 5500 rpm; Bertin Technologies), followed by the Trizol/Chloroform method as described [45]. Total RNA from BV2 was extracted with the Trizol/Chloroform method only. cDNA synthesis was carried out using the GoScript Reverse Transcription System. Quantitative PCR (qPCR) was performed as previously described [7], using the GoTaq SYBR Green mix on a StepOne Plus instrument and software (Applied Biosystems). Data normalisation was performed in comparison to the expression of human RPL-13 (section II.3.3) and, in comparison to the expression of murine RPL-19 (section II.3.4). Primer sequences are listed in

Table I.

Table I – Primer sequences

<i>Gene product</i>	<i>Primer 5'-3' Forward</i>	<i>Primer 5'-3' Reverse</i>	<i>Accession Number</i>
<i>Human RPL13</i>	CAT AGG AAG CTG GGA GCA AG	GCC CTC CAA TCA GTC TTC TG	NM_001270491.2 NM_012423.4
<i>Human TSG6</i>	TCA AGT ATG GTC AGC GTA TTC ACC	CAC AGT ATC TTC CCA CAA AGC CA	NM_007115.4 XM_054343598.1 XM_047445635.1
<i>Human IDO1</i>	GGA GAT GTC CGT AAG GTC TTG	ATA GGA GGC AGT TCC AGT TTC	NM_002164.6
<i>Mouse RPL19</i>	GAA GGT CAA AGG GAA	GAA GGT CAA AGG GAA	NM_009078.2 NM_001159483.1
<i>Mouse TNFα</i>	AGC CCC CAG TCT GTA TCC TT	GGT CAC TGT CCC AGC ATC TT	NM_013693.3 NM_001278601.1
<i>Mouse IL1β</i>	TCG CTC AGG GTC ACA AGA AA	CAT CAG AGG CAA GGA GGA AAA C	NM_008361.4
<i>Mouse iNOS</i>	AGG TAC TCA GCG TGC TCC AC	GCA CCG AAG ATA TCT TCA TG	NM_010927.4 NM_001313921.1 NM_001313922.1
<i>Mouse Arg1</i>	GGT TCT GGG AGG CCT ATC TT	TGA AAG GAG CCC TGT CTT GT	NM_007482.3

II.4. Formulation and characterization of Sph.Beads loaded with NecroX-5 nanoparticles (Sph.Beads/NP)

II.4.1. NecroX-5 nanoparticle formulation and characterization

Polymeric Nanoparticles (NPs) were synthesized by single emulsion as described previously [17, 36]. NecroX-5 (250 μ g in DMSO – 13.3 mg/mL) was added to PLGA (35mg) and PEG-PLGA (15mg) dissolved in dichloromethane (DCM) (1mL). Two mL of sodium cholate (1% w/v) were added to the mix and sonicated for 15s at 15%. The water-in-oil emulsion was added to 70mL of sodium cholate (0.3% w/v) under stirring (550rpm), and DCM was evaporated for 1h, to produce NecroX-5 loaded NP (Nx5.NP). Nx5.NP were collected by centrifugation and washed 3 times in water (10,000rpm for 30min, at 4°C). NPs were

resuspended in milliQ water and stored at 4°C. To formulate Blank NP (Blk.NP), DMSO instead of NecroX-5 solution was added to the formulation.

NPs were characterized by their size, polydispersity index (PDI) and Zeta potential (ZP) by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments). The encapsulation efficiency of Nx5.NP was measured using HPLC/UV (Shimadzu), as previously described [17].

II.4.2. Sph.Beads/NP production

Sph.Beads loaded with Nx5.NP or Blk.NP (Sph.Beads/NP) were produced as described in Section II.2.1 (5.10^6 cell/mL, alginate 0.75% and Ca-EDTA 37.5mM), with the addition of NPs in the alginate/cell suspension at 10µM (or the equivalent volume of Blk.NP). Cross-linking and bead retrieval were performed as mentioned in Section **Error! Reference source not found..** Empty alginate microbeads or encapsulating no SCAP and only NP were used as controls. Conditions are referred to as: Sph.Beads/Nx5.NP or Sph.Beads/Blk.NP when co-encapsulated with NPs, Sph.Beads for spheroid encapsulated in alginate microbeads, Beads for alginate microbeads, and Beads/Nx5.NP or Beads/Blk.NP when associated with NPs.

II.4.3. Characterization of Sph.Beads/NP

First, the incorporation of NPs in the Sph.Beads was confirmed by confocal microscopy. SCAP expressing GFP+ were co-encapsulated in alginate beads with DiD-loaded NPs (Sph.Beads/DiD.NP). Live cell nuclei staining was done using Hoechst 33342. Fluorescence microscopy images were acquired after 6, 24, and 96 hours of incubation using Z-stack acquisition (N=2, n=3). Then, NecroX-5 loading in the Sph.Beads/Nx5.NP was quantified by LC-MS after NecroX-5 extraction from the beads. Eighteen hours post-production, alginate droplets were collected (no cross-linking) in cell medium containing 10% FBS and 25mM

HEPES. Samples were centrifuged for 10 min at 10,000g and NecroX-5 was extracted as previously described [17]. NecroX-2 was used as an internal standard (60 ng/mL) (N=3 n=2). NecroX-5 release from the beads was studied as follow. Sph.Beads/Nx5.NP containing ~250,000 cells, ~5000 beads and ~80 ng of NecroX-5, were placed in Slide-A-Lyzer™ MINI Dialysis Device 2K MWCO (ThermoFischer Scientific) that were clipped on 2mL tubes containing 1.5mL cell medium, FBS10%, HEPES 25mM, and incubated at 37°C (N=2, n=3). Devices were switched onto new tubes at each time point. Release samples were stored at -20°C before analysis. One hundred µL of release medium was added to 300 µL MeOH containing internal standard (40ng/mL). Proteins were precipitated by vigorous vortexing. Then, samples were centrifuged at 14,000g for 10 min. Supernatants were concentrated by vacuum centrifugation using a CentriVap Concentrator (LabConco) (40°C – 2h). Samples were then reconstituted with 50 µL MeOH and 20 µL water, sonicated for 10 minutes, and vortexed.

LC/MS-MS quantification of NecroX-5, using NecroX-2 as internal standard, was performed by liquid chromatography coupled to tandem mass spectrometry (LC/MS-MS) as previously described using a LC/MS-MS 8040 (Shimadzu) set to a negative electrospray ionisation mode with a multiple reaction monitoring (MRM-) [17].

II.4.4. Rheological properties of Sph.Beads/NP

Sph.Beads/Blk.NP modulus was measured using a Microsquisher® (CellScale). Negative controls were Beads, Beads/Blk.NP, Sph.Beads. To approximate physiological conditions, samples were analyzed at 37°C in PBS supplemented with CaCl₂ (1.8mM) [46]. A 30sec compression ramp was applied to the samples using a 0.1524mm diameter microbeam and a 1mm² plate until a deformation of 20% was achieved. Young's modulus was determined according to **Equation 1** as described by Nativel et al. [46] (N=1, n=10).

Equation 1 – Young’s modulus calculation

$$E = \frac{\text{Stress}}{\text{Strain}} = \frac{F/A}{\Delta L/L_0}$$

Where **E** is Young’s modulus; **F** is the force applied on a particle; **A** is the area onto which the pressure is applied; **ΔL** is the displacement, and **L0** is the initial diameter of the particle.

II.4.5. Sph.Beads/NP viability

Sph.Beads/Nx5.NP cell viability was assessed by Live/Dead assay as described in Section II.3.2. Following image acquisition, quantification was carried out using ImageJ. Briefly, for each time point and condition, the mean green intensity, mean red intensity, and signal area of 20 spheroids were measured. Conditions compared Sph.Beads (as 100%) to Sph.Beads/Nx5.NP and Sph.Beads/Blk.NP after 1, 4 and 7 days in culture (N=2, n=3).

II.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism 10 software. Outliers were identified using Grubbs’ test. The Normal distribution of the data was checked before each analysis (Shapiro-Wilk test). One-way ANOVA was used when homoscedasticity was confirmed; otherwise, Welch Parametric ANOVA or non-parametric Kruskal-Wallis tests were used. Statistical tests and replicates are specified in the figure legends. Statistical significance was accepted at p-value <0.05.

III. RESULTS

III.1. *In-Bead* formation of spheroids in alginate microbeads preserved SCAP viability and immunomodulatory properties

Our objective was to obtain viable *In-Bead* SCAP spheroids in alginate microbeads using microfluidics. A new single-emulsion droplet protocol was established and optimized to produce alginate beads containing SCAP spheroids. Microfluidic alginate droplet formation was optimized to secure steady flows and reproducible droplet formation (**Supplementary Video 1**). The first challenge was to achieve homogeneous cell encapsulation throughout the production process. This was accomplished by a gentle stirring of the alginate/cell stock solution before and during the bead production, and by visually monitoring the bead cell content during the production using the system's high-speed camera. SCAP encapsulated in alginate droplets were incubated for 16, 18 and 24 hours to allow spheroid formation. Incubating for 18h was the best compromise between bead stability and cell viability (**Figure 1**).

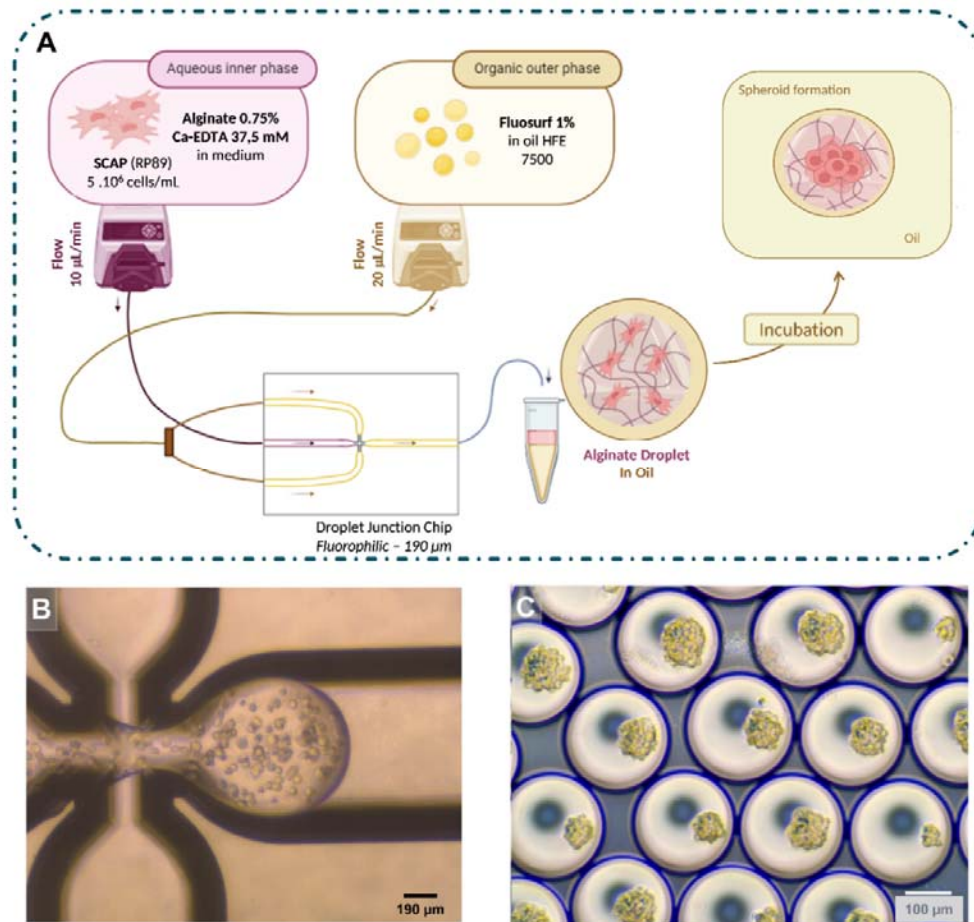


Figure 1: Encapsulation of SCAP in alginate droplets using microfluidics

A) Optimized experimental setting (BioRender). **B)** Picture of droplet generation at the chip junction ($190 \mu\text{m}$). **C)** Spheroids in alginate droplets 18h post-incubation before cross-linking.

Then, alginate droplet cross-linking and bead retrieval were optimized to reduce pH shift time, maintain cell viability, and enable single-bead cross-linking with homogeneous cell encapsulation. Internal bead cross-linking was achieved by inducing calcium release from EDTA complexes through a pH change by the addition of acetic acid (0.1%) in the oil phase (**Figure 2A**). Acetic acid exposition was limited to a minimum, while HEPES was added to buffer the solution to avoid cytotoxicity due to low pH. To further stabilize the alginate bead and prevent the spheroid from escaping during subsequent bead manipulations, CaCl_2 (40 mM in 500 μL) was added to the retrieval solution to induce post-acidic cross-linking. Beads were retrieved after the addition of PFO and medium containing Pluronic F127, used to break the

emulsion, and the oil was eliminated by pipetting. An antistatic gun was also used to help break the droplet emulsion in addition to PFO.

Sph.Beads formed after cross-linking and retrieval (**Figure 2B**) had a mean diameter of 274 μm , containing spheroids with a mean diameter of 80 μm and an average of 97 ± 24 cells (**Figure 2C**). For one sample, a theoretical number of 250,000 cells were introduced into the microfluidics system. This was confirmed by cell quantification (CI 95% 225,000-300,000). Bead formation rate was 538 ± 79 beads/min.

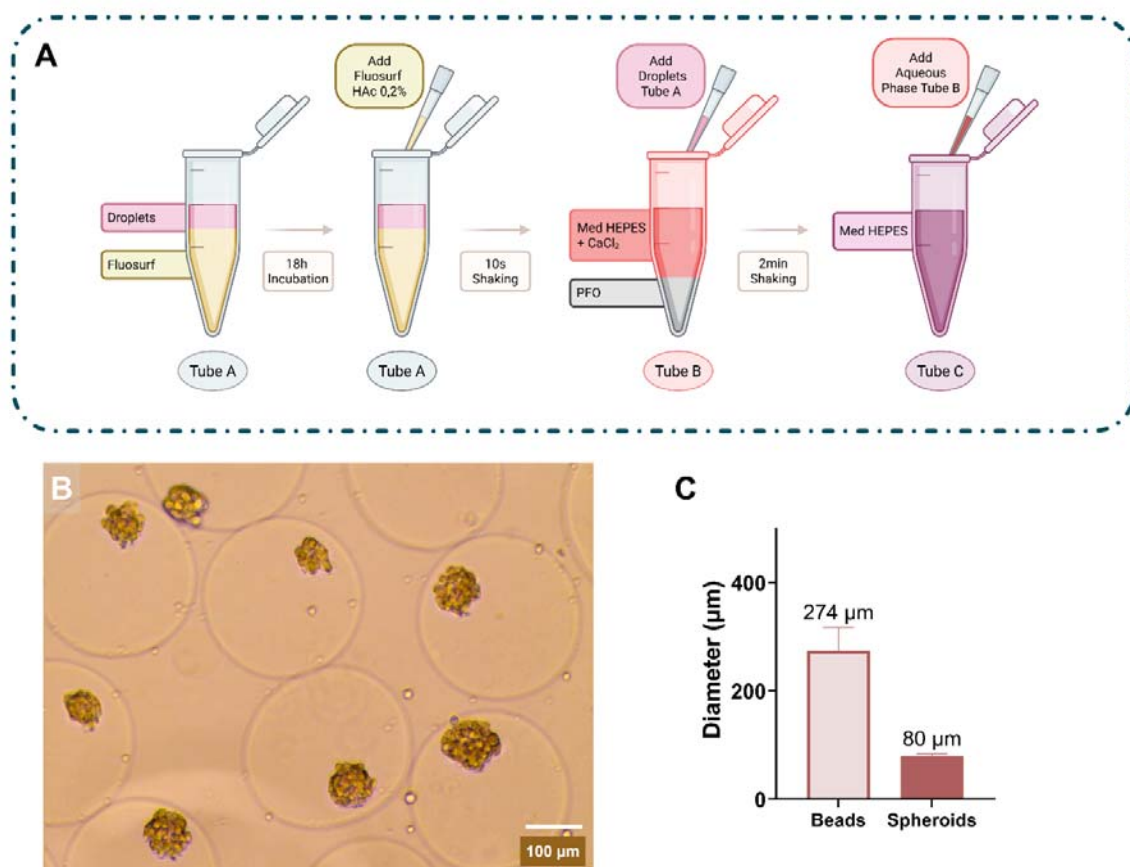


Figure 2 : Crosslinking and retrieval of Sph.beads

A) Experimental setting optimized for Sph.Bead cross-linking and retrieval (BioRender); Fluosurf HAc 0.2%: Fluosurf containing 0.2 % of acetic acid. **B)** Spheroids in alginate beads (Sph.Beads) after cross-linking. **C)** Bead and spheroid diameters measured with ImageJ (Mean $\pm$ SD, N=5 n=10).

Cell viability in Sph.Beads was confirmed using Live/Dead assay (**Figure 3A-B**). After formation, Sph.Beads exhibited a predominantly green signal across the spheroid area,

showing a high cell viability. Some cells positive to Ethidium staining were observed, indicating scarce cell death. Over time, the green signal intensity remained high, although a slight decrease was observed near the edges of the spheroid. Bright field images assessed that the bead integrity was preserved over time.

Then, the impact of a pro-inflammatory stimulus on Sph.Beads expression of immunomodulatory molecules was evaluated. TSG6 and IDO gene expression were both significantly increased upon cell activation (**Figure 3C-E**), whether SCAP spheroids were encapsulated in alginate beads or not, indicating that encapsulation did not suppress the ability of the cells to react to a pro-inflammatory priming.

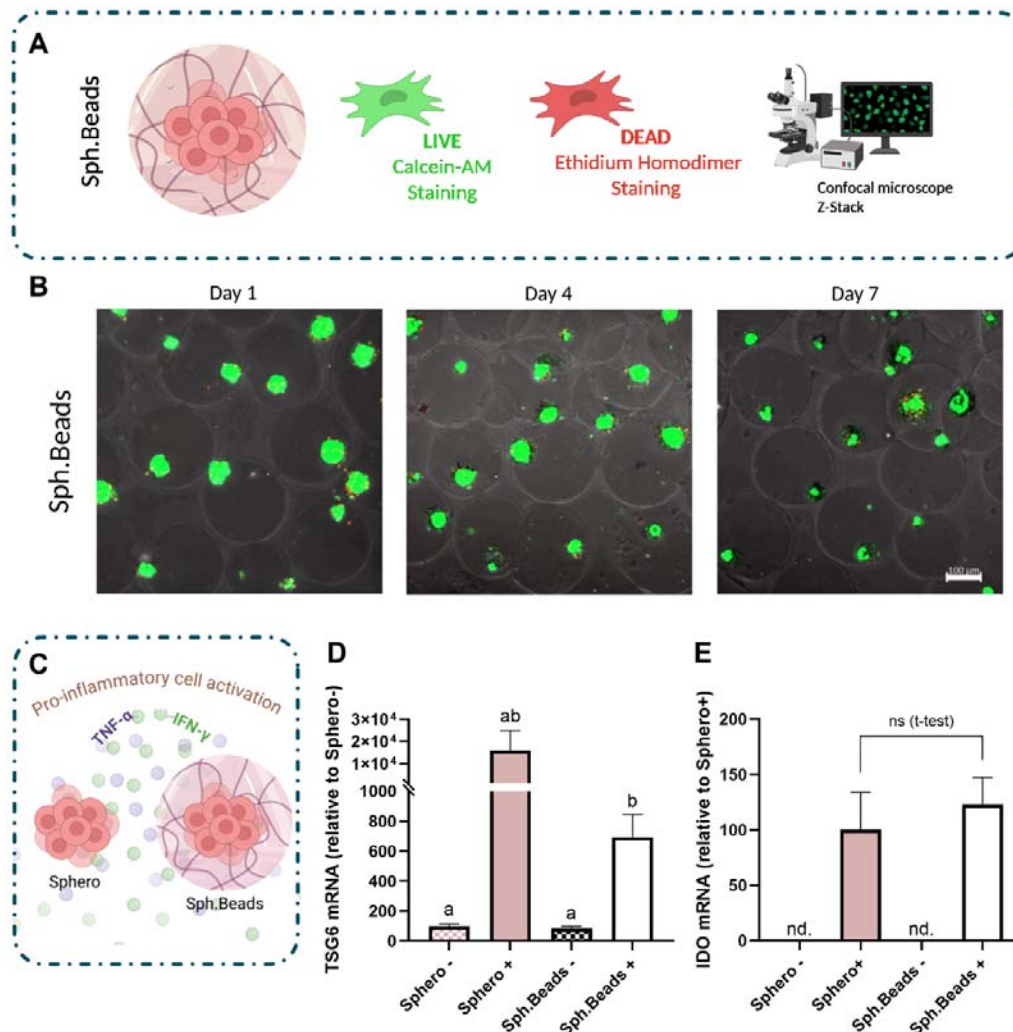


Figure 3: Characterization of spheroids in alginate microbeads

A) Experimental setting of the Sph.Beads Live/Dead assay (BioRender). **B)** Images of Sph.Beads during the Live/Dead assay over one week (Scale bar 100 μ m). Live cells are stained in green (Calcein AM), and dead cells are stained in red (Ethidium homodimer). Bright field acquisition confirmed spheroid encapsulation and bead integrity over time. **C)** Experimental setting of pro-inflammatory cell activation with $TNF\alpha$ and $IFN\gamma$ (BioRender). **D)** TSG6 mRNA expression of activated (+) or non-activated (-) spheroids and Sph.Beads (Mean $\pm$ SEM, N=2 n=3 – Welch ANOVA – Dunnet’s Test – Conditions linked by the same letter are not significantly different $p>0.05$). **E)** IDO mRNA expression of activated (+) or non-activated (-) spheroids and Sph.Beads (Mean $\pm$ SEM, N=2 n=3 – Unpaired t-test ns. $p>0.05$).

III.2. Activated BV2 treated with Sph.Beads show decreased pro-inflammatory expression

Previous studies have demonstrated that SCAP can reduce the pro-inflammatory cytokines expression of murine microglia cells (BV2) [47, 48]. Sph.Beads ability to immunomodulate its microenvironment was assessed by co-culture with activated-BV2 cells. Activation with $TNF\alpha$ and $IFN\gamma$ of BV2 microglia cells increased the expression of pro-inflammatory cytokines ($TNF\alpha$, $IL1\beta$, iNOS) compared to non-activated BV2 cells (M0). $TNF\alpha$ expression (**Figure 4**) was significantly reduced when BV2 cells were treated with Sph.Beads. $IL1\beta$ expression was not impacted by the addition of SCAP within the beads. The iNOS expression tended to diminish when BV2 cells were treated with Sph.Beads. Regarding the expression of Arginase1 (Arg1), a marker of M2 pro-resolutive microglia, expression was higher for BV2 treated with Sph.Beads.

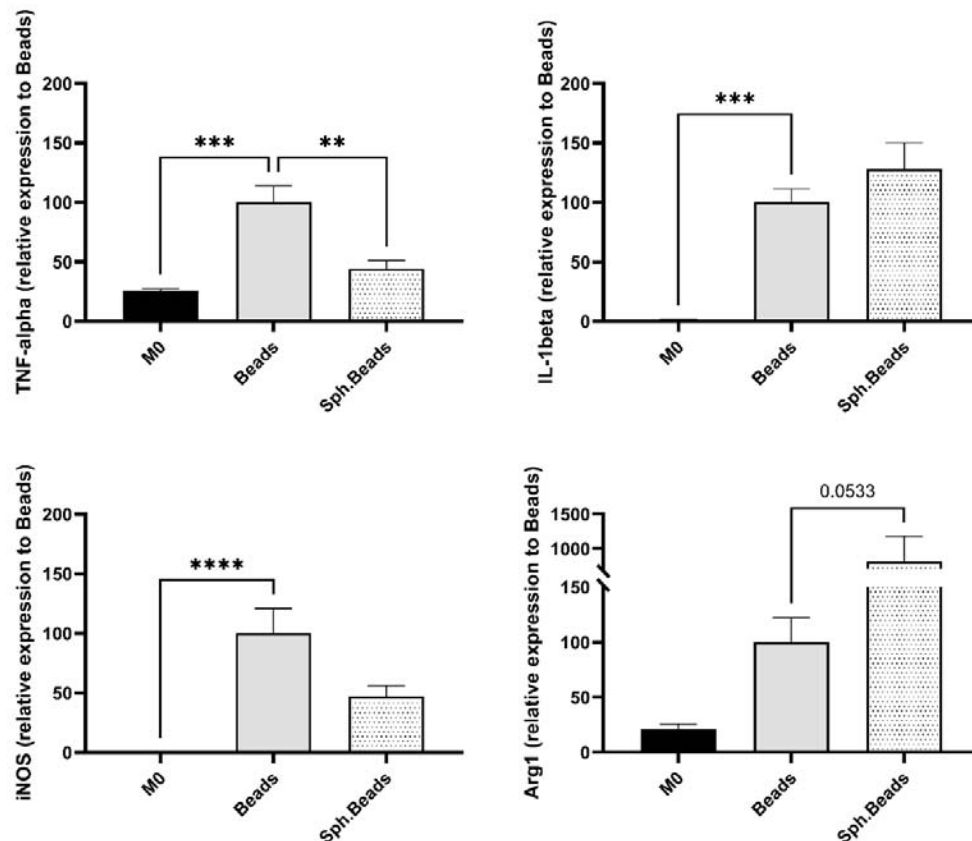


Figure 4: Impact of Sph.Beads on activated microglia gene expression

mRNA expression of pro- and anti-inflammatory markers in activated microglia (TNF α and IFN γ) treated with Sph.Beads. Non-activated microglia (M0) and activated microglia treated with empty alginate beads (Beads) were used as controls, and gene expression was normalized to Beads (Mean $\pm$ SEM – N=3 n=4– TNF α : Welch ANOVA, Dunnett's test comparison to the Beads condition, *p<0.05, **p<0.01. IL1 β , iNOS and Arg1 : Kruskal-Wallis multiple comparison and Dunn's tests comparison to CT Beads, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

III.3. Addition of NecroX-5 loaded NP in Sph.Beads (Sph.Beads/Nx5.NP) increased cell viability

III.3.1. NecroX-5 NP physico-chemical properties

NP loaded with NecroX-5 (Nx5.NP) and blank NPs (Blk.NP) exhibited similar sizes, PDI, and ZP (**Table II** – Physico-chemical characterization of NP

). NecroX-5 was encapsulated in PLGA/PLGA-PEG NPs with an encapsulation efficiency of 50%.

Table II – Physico-chemical characterization of NP

	Nx5.NP	Blk.NP
Size (nm)	237 ± 26	238 ± 21
PDI	0.12 ± 0.04	0.12 ± 0.05
ZP (mV)	-41 ± 2	-40 ± 1
Drug Encapsulation Efficiency (%)	49 ± 6	/

Data are expressed as Mean ± SD. N ≥ 10

III.3.2. Characterization of Sph.Beads/NP

The success of NP incorporation in Sph.Beads (Sph.Beads/NP) was assessed qualitatively and quantitatively. Fluorescent (DiD) NPs were added to the aqueous phase, which contained alginate and GFP+SCAP, prior to droplet formation. *In-Bead* spheroids were formed and retrieved as described in II.2. Sph.Beads/DiD.NP were imaged using confocal microscopy. DiD NPs were visible in the beads, confirming their successful incorporation (**Figure 5A-B**). They were homogeneously distributed within the beads. Over time, the DiD NP signal decreased but the NPs remained uniformly distributed within the beads. Sph.Beads/Nx5.NP were then formed using 10 µM NecroX-5. The loading efficiency of NecroX-5, as measured by LC/MS-MS, was 24% ± 4.8%, equivalent to ~2.5 µM per bead (N = 3, n = 2).

A sustained release of NecroX-5 from Sph.Beads/Nx5.NP was observed, with approximately 20% cumulative release of the loaded NecroX-5 released over 3 weeks (**Figure 5C-D**). As the initial amount of NecroX-5 in the samples was ~80 ng, ~16 ng were released. The results reflect the release of NecroX-5 from both the alginate beads and the NPs.

The Young's modulus of Sph.Beads/Nx5.NP was measured to assess the impact of NP and/or spheroid encapsulation on bead stiffness. The calculated modulus (~150 Pa) showed no significant differences between conditions (**Figure 5E-F- Supplementary Video 2**).

III.3.3. Impact of Nx5.NP on SCAP viability in Sph.Beads/Nx5.NP

The impact of the addition of Nx5.NPs in Sph.Beads on SCAP viability was evaluated using a Live/Dead assay. Compared to Sph.Beads, Sph.Beads/NP loaded with blank (Blk.NP) or NecroX-5 NPs (Nx5.NP), both showed a slight decrease in viability at Day 1, but then, at Day 4 and 7, the viability of Sph.Beads/Nx5.NP was higher than the controls (**Figure 6B&C**). The ratios of living/dead cells were higher than 10, whatever the conditions and time points, and showed a slight increase over time, except for Sph.Beads/Blk.NP (**Figure 6D**). The ratio was the highest for Sph.Beads/Nx5.NP and significantly higher than Sph.Beads/Blk.NP at Day 7.

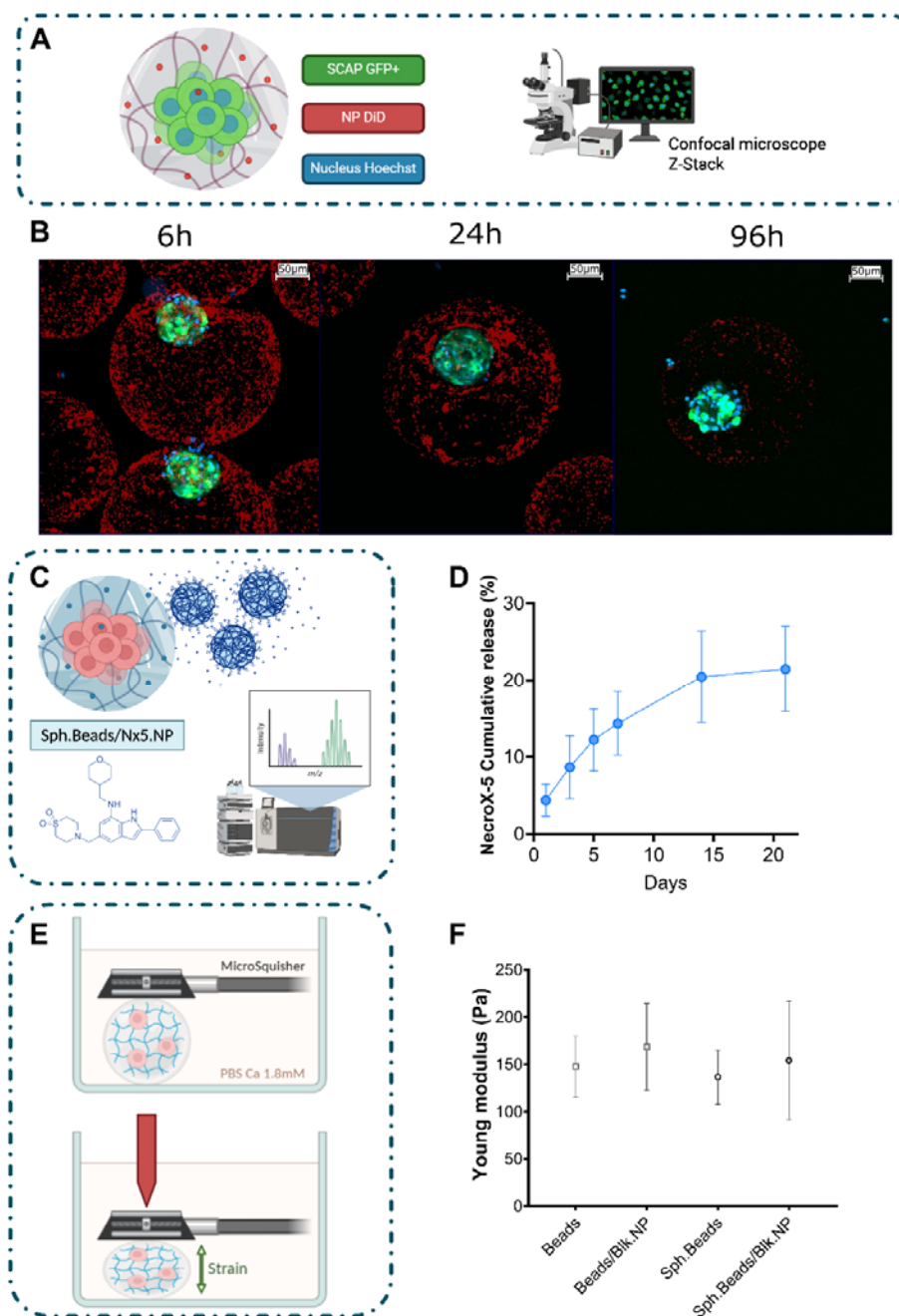


Figure 5: Characterization of Sph.Beads/NP

A) Experimental setting of confocal imaging (BioRender). **B)** NPs loaded with DiD (red) encapsulated in Sph.Beads formed with GFP+SCAP (green), with nuclei stained with Hoechst 33342 (blue) (Scale bar 50 μ m). **C)** LC/MS-MS quantification of Nx5 release (BioRender). **D)** NecroX-5 cumulative release (in %) from Sph.Beads/Nx5.NP over 21 days (Mean $\pm$ SD, N=2 n=3). **E&F)** Young's modulus measurement (Pa) of Sph.Beads/Blk.NP using a MicroSquisher (BioRender), at 37°C in PBS supplemented with 1.8 mM CaCl₂. Beads, Beads/Blk.NP and Sph.Beads were used as controls (Mean $\pm$ SD, N=1 n=10 – One-way ANOVA ns. p>0.05).

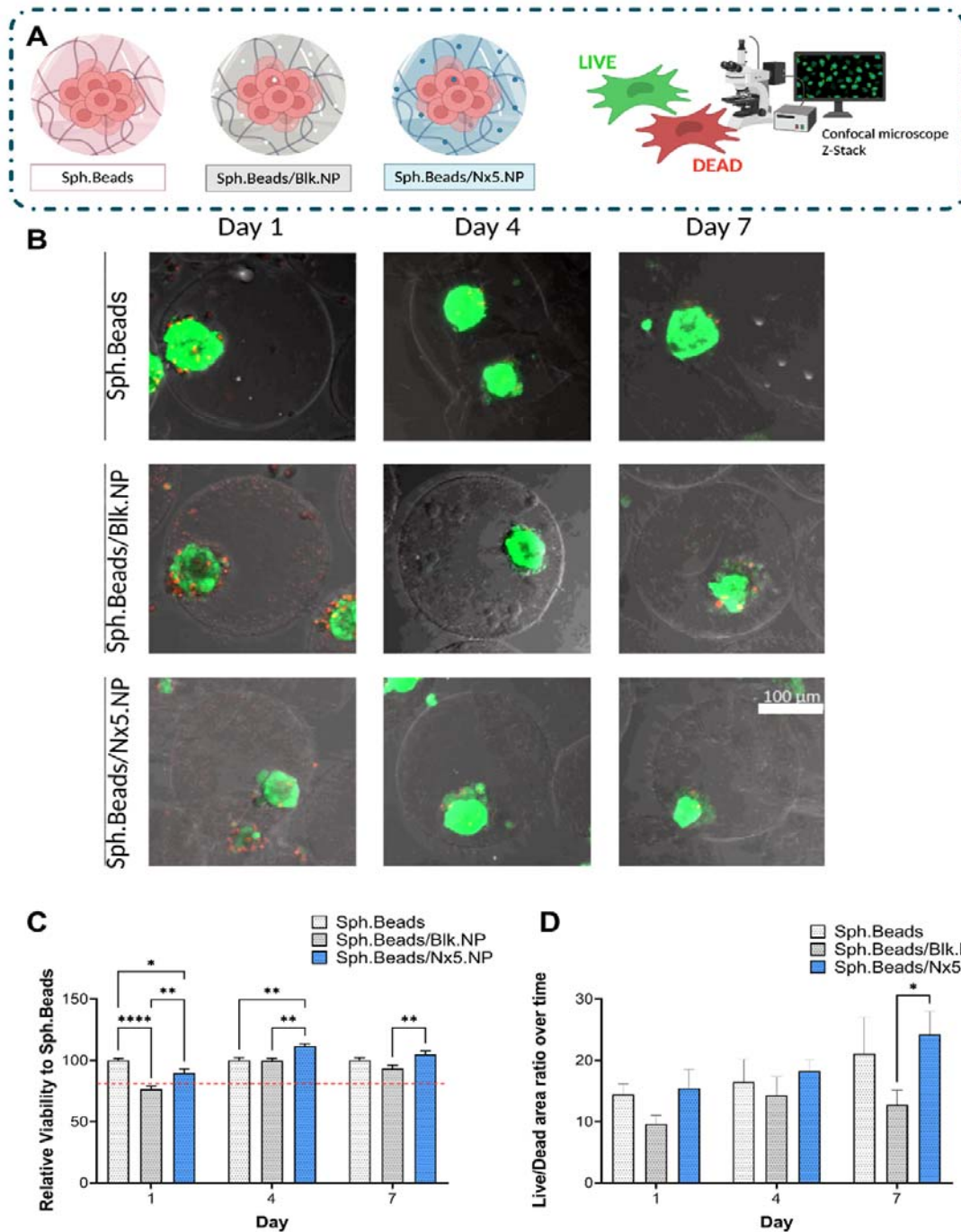


Figure 6 : SCAP viability over time in Sph.Beads/NP

A) Experimental setting (BioRender). **B)** Live/Dead picture acquisitions by confocal imaging of Sph.Beads, Sph.Beads/Blk.NP, and Sph.Beads/Nx5.NP (Scale bar 100 μ m). **C)** Spheroid viability (Green signal mean intensity) normalized to Sph.Beads over time (Mean $\pm$ SEM, N=2, n=3, 20 spheroids measured/condition – 2way ANOVA – Tukey’s tests – * p <0.05, ** p <0.01, *** p <0.001, **** p <0.0001) **D)** Ratio Living cells (area of green signal)/dead cells (area of red signal) over time (Mean $\pm$ SEM, N=2, n=3, 20 spheroids measured/condition – 2way ANOVA – Tukey’s tests – * p <0.05).

IV. DISCUSSION

The objective of this work was to develop a novel system for delivering MSC while ensuring their viability. We engineered a microfluidic-based approach to encapsulate SCAP and enable spheroid formation within alginate microbeads supplemented with nanomedicines. The primary challenge was achieving cell encapsulation using microfluidics, while simultaneously enabling cell aggregation and spheroid formation within the non-gelified alginate droplets containing nanomedicines. We succeeded in developing a process presenting a precise control of cross-linking that allowed to solidify the beads without compromising SCAP viability or the structural integrity of individual beads. Supplementation with Nx5.NP increased SCAP spheroid viability over time *in vitro*.

The initial step focused on optimizing the *In-Bead* production of spheroids (Sph.Beads) to achieve reproducible, robust formation of a single spheroid per alginate bead, while maintaining high cell viability. Using this approach, we generated beads with a diameter of 275 μm , each encapsulating a single spheroid (80 μm in diameter, containing approximately 100 cells). SCAP viability remained high throughout the production process and was sustained over one week, with only a slight decrease over time. This suggests that the production process was mild enough to preserve SCAP viability and that an effective nutrient and waste diffusion occurred, consistent with the known permeability of microbeads smaller than 500 μm [49].

This process required balancing three key factors: homogeneous encapsulation, optimal bead morphology, and spheroid retention within the bead. A delicate equilibrium had to be found between microfluidic production parameters and bead gelation and retrieval conditions to ensure both SCAP viability and retention of the spheroid within the beads. To address these challenges, we integrated several approaches to i) produce beads smaller than 300 μm in diameter, ensuring injectability in rodent models (21G needle), ii) stabilize the emulsion long

enough to allow *In-Bead* spheroid formation (18h), iii) develop a tailored gelation protocol (combining calcium release from EDTA complexes by the addition of acetic acid, HEPES to buffer the solution and CaCl_2 to induce post-acidic cross-linking) and retrieval to solidify beads without compromising cell viability or structural integrity (combination of PFO and medium containing Pluronic F127 and an antistatic gun). This protocol addresses all these constraints simultaneously, enabling the production of *In-Bead* spheroids combined with nanomedicines. While microfluidic spheroid formation has gained attraction in oncology for high-throughput screening [50, 51], the behavior of cancer cells, which can rapidly proliferate and form spheroids, differs significantly from that of MSCs. This discrepancy complicates the direct translation of cancer cell protocols to MSC applications, which explains the need for protocols specifically optimized for MSC spheroid formation.

While microfluidic techniques are well established for cell encapsulation in hydrogels, including MSCs [52, 53], and for spheroid formation, the integration of these two processes, particularly the formation of spheroids within alginate microbeads, remains rarely documented as to our knowledge, only one other group, Chan et al., has described a double emulsion microfluidic protocol for MSC *In-Bead* spheroid formation [54]. Their method relied on custom-designed microfluidic systems and required two sequential chips to generate the double emulsion, necessitating the control of three fluid phases. These features increase operational variability and enable oil entrapment within W/O/W droplets, complicating complete oil removal compared to a single emulsion protocol. In this study, we developed a protocol using a commercial microfluidic system with a commercially available chip, providing a standardized and accessible platform for single emulsion droplet generation and cell/NP encapsulation. Our novel approach overcomes reproducibility challenges. Here, building on automated spheroid formation techniques described by Langer et al. [43], we optimized for the first time a single-step, in-situ process for *In-Bead* spheroid formation. This

required precise control of droplet stability and cell aggregation to prevent premature spheroid leakage or gelation. A critical innovation was triggering external gelation without disrupting droplet integrity, enabling recovery of round, non-aggregated beads containing *In-Bead* formed spheroids.

We then demonstrated that the Sph.Bead formulation did not compromise SCAP responsiveness to a proinflammatory stimulus. Previously, we showed that SCAP, whether as single cells or spheroids (formed via magnetic bioprinting), upregulated immunomodulatory molecules such as TSG-6 and IDO upon activation with TNF α and IFN γ [17, 44]. In this study, we confirmed that the formation of *In-Bead* spheroids did not impair this response, as activated SCAP spheroids maintained elevated TSG-6 and IDO expression. This suggests that TNF α and IFN γ (50 and 20 kDa, respectively) effectively diffused through the alginate matrix to reach the spheroid and that encapsulated spheroids were still able to react to this stimulus [55]. Furthermore, when Sph.Beads were co-cultured with activated microglial cells, they suppressed pro-inflammatory markers (TNF α , iNOS) while upregulating the anti-inflammatory marker Arg1 in activated microglial cells, indicating a shift toward an M2-like phenotype. These results highlight the ability of Sph.Beads to preserve and recapitulate the immunomodulatory properties of both SCAP cells and spheroids.

To further enhance Sph.Bead functionality, we incorporated NecroX-5 nanoparticles (Nx5.NP) during droplet formation, producing Sph.Beads/Nx5.NP. The Nx5.NP, prepared as previously described [17], were uniformly distributed within the beads, demonstrating for the reproducible loading of nanomedicines into microgels using our microfluidic protocol. A time-dependent decrease in fluorescence intensity suggested NP release, which was confirmed by quantification, revealing sustained release kinetics. However, the final Nx5.NP loading in the Sph.Beads was $\sim 2.5 \mu\text{M}$, reflecting a 75% loss from the initial $10 \mu\text{M}$ concentration in the alginate stock solution. We thus hypothesized that during the 18-hour incubation enabling

spheroid formation, Nx5 diffused from the NPs into the non-gelled alginate and partitioned into the oily phase, driven by equilibrium distribution. This was supported by experiments where free NecroX-5 was encapsulated in alginate droplets under identical conditions, which resulted in negligible drug retention in the beads (Supplementary Figure 1). The absence of an observable burst release in our study may be explained by an initial burst during this incubation, consistent with findings by Guo et al., who reported a 75% burst release of Coumarin-6 (a lipophilic molecule) from PLGA NPs within 4 hours when encapsulated in PEG-alginate beads [56]. This underscores a challenge in retaining lipophilic drugs during oil-based microfluidic encapsulation. Despite this loss, the residual Nx5 concentration ($\sim 2.5 \mu\text{M}$) remained within a therapeutically relevant range, as NecroX-5 has shown efficacy against oxidative stress at concentrations as low as $0.1 \mu\text{M}$ and protected hepatocytes from doxorubicin toxicity at $0.125 \mu\text{M}$ [34], well below the levels typically used in vitro [57-59]. Thus, even with reduced loading, the Sph.Beads/Nx5.NP may retain biological activity.

The Young's modulus of the Sph.Beads/NP was approximately 150 Pa, with neither *In-Bead* spheroid formation nor NP encapsulation significantly altering mechanical properties. Hydrogel stiffness is a critical parameter, as it influences mechanotransduction and stem cell behavior; softer gels are known to preserve stemness [60]. Alginate microgels typically exhibit moduli ranging from 0.2 to 20 kPa, depending on the cross-linking agent, providing a soft 3D environment that supports cell-cell interactions and culture conditions [60]. Our results align with findings by Cappello et al., who reported a Young's modulus of ~ 100 Pa for $200 \mu\text{m}$ beads fabricated from 1% low-viscosity alginate using microfluidics [61]. This consistency confirms that our system maintains optimal mechanical properties for stem cell encapsulation and spheroid culture.

For the first time, we demonstrated that incorporating Nx5.NPs into Sph.Beads enhanced cell viability. While a slight viability decrease was observed at Day 1 for both Sph.Beads/Nx5.NP

and Sph.Beads/Blk.NP, likely due to high NP loading approaching cellular tolerance thresholds, viability significantly improved by Day 4 in the Nx5.NP group. This supports the protective role of NecroX-5, though its effect might be more pronounced at concentrations closer to 10 μ M. However, simply increasing NP content is not viable, as it compromises Day 1 viability and risks altering alginate viscosity or NP stability. Since improving encapsulation efficiency to boost Nx5 payload per NP proved challenging, future work could explore alternative nanocarriers or hydrophilic therapeutics (e.g. RNA-based drugs), where diffusion losses may be less critical. This system thus holds promise for broader applications beyond lipophilic compounds.

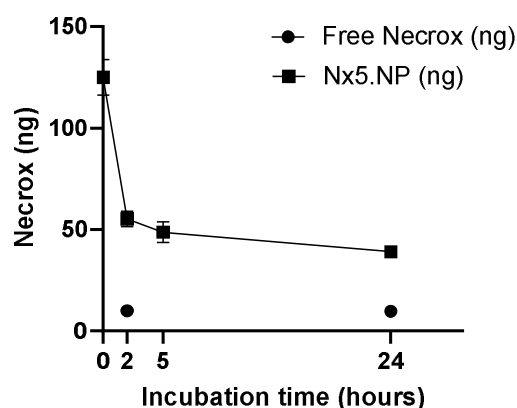
V. CONCLUSION

In this study, we established a novel microfluidic protocol for the *In-Bead* generation of MSC spheroids within nanomedicine-loaded alginate microbeads. This approach combines reproducibility, scalability, and therapeutic innovation, enabling the seamless integration of nanomedicines with spheroid-laden beads. By incorporating NecroX-5-loaded nanoparticles, we demonstrated a modest but significant enhancement in SCAP viability, highlighting the potential of this dual-delivery strategy. While further optimization, particularly in nanoparticle retention and drug loading efficiency, is needed, our findings provide critical insights into the synergy between nanomedicines and MSC-based therapies. This platform lays the foundation for targeted regenerative medicine applications, where refined delivery strategies could substantially improve MSC survival and therapeutic outcomes.

Acknowledgements

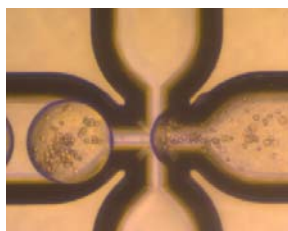
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VI. SUPPLEMENTARY MATERIALS



Supplementary Figure 1: Impact of droplet incubation time on NecroX-5 loading

Quantification of free NecroX-5 versus encapsulated NecroX-5 by LC/MS-MS based on the incubation time within the droplets before retrieval (N=1 n=2).



Supplementary Video 1: Microfluidic droplet generation

High-speed camera video of the production of droplets using the Dolomite™ microfluidics system with a 190 μm fluorophilic chip. SCAP introduced at 5.10^6 cells/mL in alginate solution (0.75% - CaEDTA 37.5mM) with Blk.NP with a flow rate of 10 $\mu\text{L}/\text{min}$. Organic phase HFE 7500 with 1% Fluosurf at 20 $\mu\text{L}/\text{min}$.



Supplementary Video 2: Microsquisher – Sph.Beads/Blk.NP

Images acquired using the Microsquisher® (CellScale). The force required to achieve a 20% displacement was recorded. Bead containing a SCAP spheroid and Blk.NP presented in this video.

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