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Biomimetic microspheres for 3D mesenchymal stem cell culture and characterization

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Abstract

Stem cells reside in niches, specialized microenvironments that sustain and regulate their fate. Extracellular matrix (ECM), paracrine factors or other cells are key niche regulating elements. As the conventional 2D cell culture lacks these elements, it can alter the properties of naïve stem cells. In this work we designed a novel biomimetic microenvironment for cell culture, consisting of magnetic microspheres, prepared with acrylates and acrylic acid copolymers and functionalized with fibronectin or hyaluronic acid as ECM coatings. To characterize cell proliferation and adhesion, porcine mesenchymal stem cells (MSCs) were grown with the different microspheres. The results showed that the 3D environments presented similar proliferation to the 2D culture and that fibronectin allows cell adhesion, while hyaluronic acid hinders it. In the 3D environments, cells reorganize the microspheres to grow in aggregates, highlighting the advantages of microspheres as 3D environments and allowing the cells to adapt the environment to their requirements.

Keywords

3D environment, extracellular matrix coatings, microspheres, mesenchymal stem cells, surface modification

1. Introduction

Stem cell therapies have recently gained importance in the field of regenerative medicine; MSC have received special attention for their immunomodulatory properties, their ability to differentiate between several phenotypes and to secrete paracrine factors. [1] For example, the therapeutic benefits of MSC have been tested in different clinical approaches for hematologic, cardiovascular, neurological and bone and cartilage disease, as well as for graft versus host disease. [2] However, MSC need to

be expanded ex vivo prior to use in cell-based therapies, since they have a very scant population in tissues, which makes it impossible to reach the high cell volumes required for therapeutic applications. [3]

In vivo, stem cells reside in specialized microenvironments, termed *niches*, which are microenvironments that sustain and regulate them. The support or niche cells, the physical signals, the ECM components, and hormonal and paracrine factors are key elements in maintaining stem cell homeostasis and consequently in determining stem cell fate. [4]

The conventional technique of 2D culture can thus alter the inherited properties of the naïve MSC, which originally reside in a 3D environment with the relevant role of the ECM, key in regulating stem cell fate decisions, such as proliferation, differentiation and self-renewal.[5] Despite the amazing advances in the field of cell culture, our ability to translate biological insights has thus been mitigated because bi-dimensional culture on plastic plates is very different from the in vivo microenvironment. Cells not only change their behavior in this non-physiological environment, but are also unable to remodel the ECM and reorganize it freely, as they would during development and homeostasis. [6] These limitations indicate the need to develop innovative platforms able to provide more biomimetic conditions for cell culture. These artificial analogs of the in vivo niches could become a primary platform for preclinical testing, and play a major role in our basic understanding of stem cell biology with direct clinical applications.

Biomaterials are deemed necessary in this direction. Attempts to achieve a more biomimetic culture environment for MSC and cells in general have been made by simply providing 3D platforms like scaffolds or hydrogels, some of them including ECM components. For example, Di Maggio et al. tried to model the bone marrow niche of MSC and hematopoietic stem cells (HSC) on a 3D perfused scaffold that could be

subjected to different molecular functionalizations.[7] Silva et al. studied the modulation of MSC secretome when cultured in hydrogels modified with fibronectin peptides.[8] Although studies using polymeric microspheres are now becoming more common, these platforms are often used in low-density cultures as microcarriers or as cell microencapsulation devices for cell expansion or delivery, but not as substitute 3D cell biomimetic culture environments for scaffolds or hydrogels.[9,10]

We believe a 3D environment defined by agglomerated microspheres has advantages over more conventional tissue engineering approaches, like the aforementioned scaffolds or hydrogels. It is an operationally more versatile system which, for example, allows to separate and recover cells from the support (when working with magnetic microspheres) after finishing the culture, which would be very useful in cell culture studies. The possibility of using different ECM molecule coatings provides the tools to control cellular fate by rational manipulation of the niche and offers a more biomimetic material. Also, agglomerated microspheres have other more important advantages: they constitute a more flexible 3D environment than scaffolds and hydrogels, as microspheres can be reorganized by cells during the culture to lodge their own secreted ECM. Cells can establish cell-cell and cell-matrix interactions in all spatial directions without the physical restrictions imposed by a rigid and non-malleable support (like a scaffold), or without being trapped in an isolated location (as in a hydrogel). They offer the opportunity to go from a 2D environment (low microsphere density in the culture) to a 3D culture (higher microsphere densities), in which cells can develop cell-cell and/or cell-matrix interactions simply by increasing the number of microspheres (**Figure 1**).

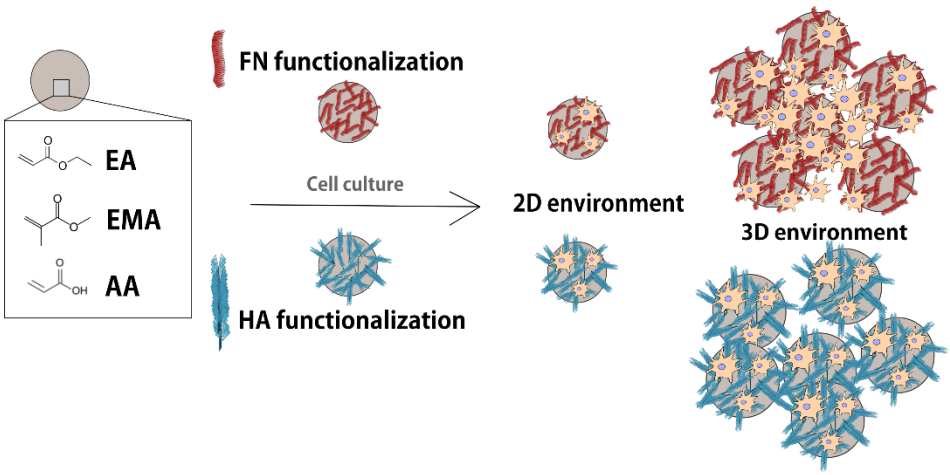

Figure 1. Representation of the 3D biomimetic microsphere-based cell culture environments tested. EA: ethyl acrylate, EMA: ethyl methacrylate, AA: acrylic acid, FN: fibronectin, HA: hyaluronic acid.

The aim of the present study was to develop a new 3D biomimetic cell culture platform formed by agglomerated microspheres. Microspheres are magnetic, and their composition is specially designed to be biostable and compatible with different molecular functionalizations, like ECM molecule coatings, specifically fibronectin (FN) and hyaluronic acid (HA), giving a more suitable biosimilar context for MSC culture. The physicochemical properties of a given biomaterial modulate biomolecule adsorption on its surface and play an important role in determining the design of the biomaterial. The chemical composition of the copolymers used in this work was designed on the basis of the specific properties of each monomer. Poly (ethyl acrylate) p(EA) has been shown to induce FN fibrillogenesis in the absence of cellular activity, which is the origin of this polymer's good performance in 3D cultures.[11,12] However, as the resulting polymer gave rise to microspheres that were sticky and difficult to handle, ethyl methacrylate (EMA) was incorporated in the copolymer to improve their properties, increase the glass transition temperature, T_g, and make the microspheres easier to handle. The glass transiton temperature measured in the copolymer of EA and EMA by DSC is 24.3ºC. Acrylic acid (AA) was incorporated in the copolymer to introduce acid functional groups to the microsphere surface to facilitate further

functionalizations via the amide bond, the glass transiton temperature measured in the copolymer of EA, EMA and AA by DSC is 22.8ºC. According to the reference[13] a low glass transition temperature, Tg, of the support is a requirement for fibrillogenesis and even if the Tg of our copolymers is below 37ºC, FN fibrillogenesis is unlikely to be induced. However, the good biocompatibility of the copolymers and their extended use in the biomedical field makes this selection a good candidate for the cell culture platform.

FN is a dimeric glycoprotein, which is one of the main components of the ECM, and modulates several processes, like cellular adhesion, differentiation or proliferation via direct interactions with cell surface integrin receptors, such as α5β1 integrin. It also interacts with other FN molecules, different ECM components and growth factors (GFs), has an impact on tissue organization and contributes to ECM assembly.[14,15] The FN matrix is key for normal cell adhesion and growth and so was chosen for coating the microspheres.[16] This biomimetic modification was expected to enhance cell adhesion and boost natural ECM development in the 3D platform. HA is an anionic and linear glycosaminoglycan that occurs naturally in the ECM, with key roles in embryonic development, tissue organization, wound healing, angiogenesis, tumorigenesis and even in determining biomechanical tissue properties.[17] It is well known that HA binds to cell-surface receptors, such as CD44, and contributes to regulating cell motility and adhesion, however, unlike FN, HA promotes migration and can also inhibit cell adhesion and also plays a key role in ECM remodeling.[18] It was therefore chosen for testing as a biomimetic ECM coating in the microspheres with opposite properties to those of FN.

The behavior of porcine MSCs (pMSC) was evaluated in terms of cellular proliferation cultured in the resulting 3D platform using fibronectin or hyaluronic acid as coating for the developed microspheres (**Figure 1**).

2. Materials and Methods

2.1. Polymerization process

Two different polymeric materials were synthesized with different proportions of EA(Sigma-Aldrich), EMA (Sigma-Aldrich) and AA (Scharlau). The first material, p(EA-EMA-0%AA), was composed of 50% EA, 50% EMA and 0% AA; and the second, p(EA-EMA-5%AA), of 47.5% EA, 47.5% EMA and 5% AA. The polymerization was a free radical reaction, achieved by mixing all the monomers in the desired proportion and using acetone 30 wt% (Scharlau) as solvent and benzoin 0.5 wt% (Sigma-Aldrich) as initiator. The resulting solution was polymerized for 24 hours in an ultraviolet oven. The polymeric blocks thus obtained underwent a thermal post-polymerization process for 24 hours at 90ºC in another oven (Memmert GmbH + Co.KG, Germany) to ensure total monomer conversion. After polymerization the remaining low molecular weight substances were extracted by repeated dissolution and precipitation, for which the copolymers were diluted in acetone, re-precipitated with deionized water and then dried for 3 days at 60ºC in oven, repeating the process three times.

2.2. Microsphere production

Microspheres were produced via an oil/water (o/w) emulsion method with solvent evaporation. [19] The aqueous phase consisted of a solution of polyvinylalcohol (PVA, Sigma-Aldrich) in MiliQ water (4% w/v) and the organic phase was the polymer dissolved in chloroform (Scharlau) (3% w/v). Ferrite nanoparticles (MNPs, EMG1300 Ferrotec Ferrofluids, nominal particle diameter: 10 nm) were added to the organic phase to confer magnetic properties on the resulting microspheres, MNPs were added at 5% w/w with respect to the polymer weight. 20 mL of the polymer solution was added using a syringe pump (New Era Pump Systems Inc, USA) with a flow rate of 1mL/min in 200 mL of aqueous phase under 150 rpm agitation (IKA Works Inc, Germany).

Fifteen minutes after the polymer incorporation 150 mL of miliQ water was added, after which the emulsion was stirred for 48 hours. The microspheres were then washed four times with MiliQ water by decanting the suspension, the water was removed and the microspheres were frozen gradually to -80ºC for subsequent lyophilization for 48 hours in a LyoQuest 85 (TELSTAR, Spain).

2.3 Field emission scanning electron microscopy and diameter distribution study

The morphology of the microspheres was observed under a field emission scanning electron microscope (FESEM) Ultra 55 (Zeiss Auriga Compact, Germany). Images were taken at 1 kV with previous platinum sputter coating by a JF1100 (JEOL device, Japan). Images of the dry microspheres were taken by an MZ APO stereo microscope (Leica Microsystems, Germany) to study diameter distribution and processed for automatic diameter measurement by ImageJ software (National Institutes of Health, USA).

2.4 Microsphere functionalization and characterization

2.4.1 Hyaluronic acid functionalization

Before functionalization, the molecular weight of hyaluronic acid (HA, Sigma-Aldrich) was reduced from 1,06 MDa to 320000 Da by acidic degradation.[20,21] HA grafting onto the microspheres was based on the activation of the carboxyl groups (COOH) of the material using 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDC, Sigma-Aldrich) in combination with N-hydroxysuccinimide (NHS, Sigma-Aldrich), as in previous studies.[22–24] Activated COOH groups of AA molecules in the microspheres were made to react at one end with the amine groups of a molecular bridge, a di-amine

terminated poly(ethylene glycol), forming an amide bond. The free amine groups at the other end of the bridge were then made to react with the COOH groups of HA by the same chemical reaction. For the first COOH activation, 150 mg of AA containing microspheres were incubated for 2 hours at room temperature (RT) in EDC (1% w/v) and NHS (0.1% w/v) in Dulbecco's Phosphate-Buffered Saline (DPBS, Sigma), 3 mL of solution was used to ensure that all the microspheres were submerged. Activated microspheres were then incubated for 24 hours at 37ºC in 3 mL of a 20mM solution of poly (ethylene glycol)bis(3-aminipropyl) terminated (PEG-di-NH_2, Sigma-Aldrich) in DPBS followed by 3 washes in DPBS. In order to pre-activate the COOH groups of HA, the polymer was dissolved at 5% w/v at RT in DPBS and incubated for 2 hours at RT in EDC (1% w/v) and NHS (0.1 vol%). PEG-di-NH_2 grafted microspheres were then incubated for 24 hours at 37ºC with 3 mL of the pre-activated HA solution. The microspheres were washed with a solution of citric acid (Sigma-Aldrich) (0.5 M) and TritonX-100 (Sigma-Aldrich) (0.1vol%) due to its antibacterial and antimycotic properties and then twice with distilled water. [24] Finally, unreacted COOH groups were deactivated by incubating the microspheres in 3 mL of ethanolamine (Sigma) (1M, pH 9) for 1 hour at 4ºC followed by 3 washes with distilled water. [26]

The amount of HA grafted onto the microsphere surfaces was determined by the indirect colorimetric method of toluidine blue (TB, Sigma Aldrich). [27] TB (0.005 %w/v) was dissolved in hydrochloric acid (Sigma-Aldrich) (0.01 M) containing sodium chloride (NaCl, Panreac) (0.2% w/v). Standards of known HA concentrations were prepared in DPBS. 120 µL of sample (functionalized microspheres suspensions) or standards were mixed with 60 µL of TB solution and incubated for 30 minutes in darkness with stirring at RT so that the TB and HA could form a complex. This complex was then removed to allow measurement of the unbound TB. In the case of the samples, the complex functionalized microspheres-TB was removed by a magnet. In the case of the

standards, n-hexane (VWR) was added (60 µL/sample) for phase separation, samples were stirred vigorously and the TB-HA complex was extracted with the organic layer while the unbound TB remained in the aqueous phase for the measurement. In both cases the unbound TB was quantified by measuring the absorbance of the aqueous solution at 631nm on a UV-Vis Cary 60 spectrophotometer (Agilent Technologies, USA). The linear relationship between the absorbance at 631 nm caused by the unbound toluidine blue and the concentration of HA was ascertained from the standards measurements and used as a calibration curve. For the samples, the amount of immobilized HA was then calculated from the previously established calibration curve.

2.4.2 Fibronectin functionalization

Fibronectin (FN) functionalization was achieved by physisorption. As this process took place just before cell seeding, lyophilized microspheres were firstly conditioned and disinfected (Section *2.5.1.1)*. A coating with FN from human plasma (Sigma-Aldrich) (20µg/mL in DPBS) was performed by incubation at RT for 1 hour, after which the microspheres were washed twice with DPBS. [12]

After adsorption, the amount of FN adsorbed onto the microsphere surfaces was assessed by two techniques: the *Micro BCA™ protein assay kit* (Thermo Scientific) and SDS-PAGE in combination with image analysis; and estimated as the difference between the total FN used in the functionalization and that remaining in the supernatant recovered after the coating, plus that adsorbed by the walls of the Eppendorf coating recipient.

2.4.2.1 BCA assay

The standard protocol provided with the kit was used. Three replicates were prepared from each sample and each replicate was read 3 times by a Victor 3 plate reader (Perkin Elmer, USA).

2.4.2.2 SDS-PAGE

SDS-electrophoresis was performed to discriminate FN from other proteins present in the FBS used in the microsphere conditioning step. An 8% polyacrylamide gel was used [4.6 mL of MiliQ water, 2.6 mL of Tris-HCl (Sigma-Aldrich) (1.5M, pH8.8), 0.1 mL of SDS (Sigma-Aldrich) (10% w/v), 2.6 mL of acrylamide/bys-acrilamide (Sigma-Aldrich) (30%/0.8% w/v), 0.1 mL of ammonium persulfate (Sigma-Aldrich) (10 vol%) and 0.01 mL of TEMED (Sigma-Aldrich)]. Samples were prepared in loading buffer [β-mercaptoetanol (Sigma-Aldrich) (10mM), Tris-HCl (0.2M, pH 6.8), glycerol (20 vol%), bromophenolblue (Sigma-Aldrich) (0.05% w/v), SDS (10% w/v)] at a ratio of 5:1 and incubated for 7 minutes at 100ºC. Electrophoresis was carried out for 30 minutes at 10 V/gel and 1 hour at 20 V/gel using the running buffer [Tris-HCl (25 mM), glycine (Sigma-Aldrich) (200mM), SDS (0.1% w/v)] (BIO-RAD). The gel was stained by staining solution [Coomassie blue (0.1 vol%), methanol (50 vol%), acetic acid (10 vol%)] for 20 minutes at RT while stirring. The gels were finally washed with destaining solution [methanol (40 vol%), acetic acid (10 vol%] and visualized by a transilluminator (BIO-RAD, USA). The digital analysis of the resulting images was performed on Image Studio Lite software Version 5.2 (LI-COR Biosciences, USA).

2.5 Cell culture assays

2.5.1 Porcine mesenchymal stem cell culture

Porcine MSCs were used in the cell culture assays. [28] For cell expansion and culture DMEM high Glucose with Glutamax media was used (Fisher) supplemented by 10

vol% FBS (Fisher) and 1 vol% P/S (Gibco, 10.000 U/mL). All the cell culture essays were performed with cells at passage 6.

2.5.1.1 Cell culture seeding conditions

Microsphere suspensions from the different polymers and with different coatings were disinfected by immersion in antibiotic and antifungal solutions; penicillin/streptomycin 3vol% with amphotericin B (Sigma, 250 µg/mL) 0.5 vol% and penicillin/streptomycin 1 vol% with amphotericin B 0.5 vol% consecutively. The microspheres were then cleaned twice with DPBS.

The volume of microsphere suspension containing 10 mg of magnetic microspheres was placed in an Eppendorf tube and allowed to equilibrate for 48 hours in cell culture media with 10 vol% FBS and 1 vol% of P/S in static conditions in an incubator at 37ºC and 5% CO_2 for each 3D sample. Tissue culture polystyrene (TCPS) wells were used as 2D controls.$1x10^5$ cells were harvested per 10mg of microspheres in the 3D samples and $5x10^3$ cells/cm^2 in the 2D controls. To optimize adhesion in the 3D samples the cells were firstly harvested for 2 hours in the incubator (37ºC, 5% CO_2) within the microsphere pellet but with only 20 µL of cell culture media, after which 500 µL of culture media was added and culture time started (**Figure 2**).

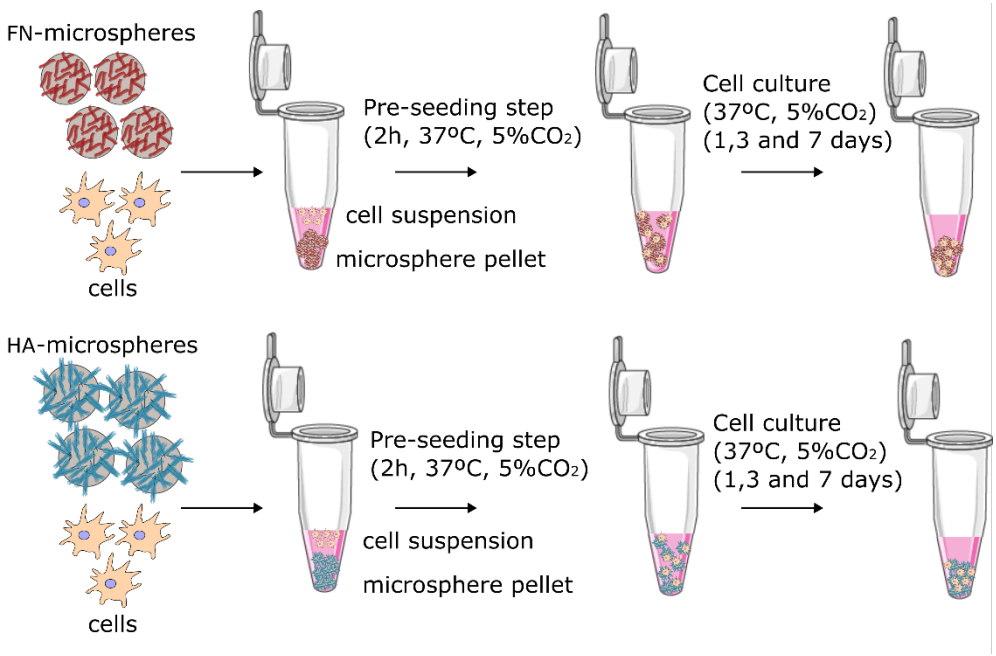

Figure 2. Scheme of the seeding procedure. (FN: Fibronectin, HA: Hyaluronic Acid).

2.5.2 Proliferation essay (*PicoGreen*)

Porcine MSC proliferation was assessed in the 3D environments and in the 2D control using the Quant-iT PicoGreen™ dsDNA commercial kit (Thermo Fisher Scientific) at 1,3 and 7 days of culture. Before analysis, to make DNA accessible, cell digestion of the samples was performed with proteinase K (Roche) (50 µg/mL, pH8) in DPBS by 16 hours incubation at 60ºC. The samples were then incubated for 10 minutes at 90 ºC for enzyme inactivation and centrifuged (1 minute, 650 rpm) to favor deposition of cellular debris and material in the bottom of the Eppendorf while the DNA remained in the supernatants. DNA was incubated with the PicoGreen solution, following the manufacturer's instructions and analyzed on an opaque plate Optiplate96F (Perkin Elmer) using a Victor3 plate reader (Perkin Elmer, USA) at 485/535 nm. The lambda phage standards provided in the kit were used for the calibration curve. The obtained absorbance data were converted to % proliferation (% of cell density increase with reference to initial cell density) in order to normalize the data, using the ascertained calibration curve, and considering 9.55 µg of DNA/cell (data ascertained in previous

experiments carried out with these cells in our lab, data not shown), followed by the statistical analysis of the data. Each condition was analyzed with 3 replicates and each replicate was read 3 times. After confirmation of data normality (D'Agostino and Pearson's test) statistical significance was assessed by an ANOVA-One Way Analysis (Tukey's test) with a 95% confidence interval using GraphPad Prism7.0 (GraphPad Software Inc., USA).

2.5.3 Flow cytometry (cell cycle assessment)

The cell cycle was analyzed by flow cytometry in cells cultured in the different 3D environments and in the 2D control at 1, 3 and 7 days. Synchronization in the G0 stage was achieved before cell seeding by culturing the cells in low serum medium (0.5 vol% FBS) for 12 hours. Cells were released back into cycle 2 hours before cell seeding by replacing the low serum medium by normal serum medium containing 10 vol% FBS. After the culture period, the cells were detached from the material by trypsinization. Briefly, samples were washed with DPBS, then 300 µL Trypsin-EDTA 1X (Invitrogen) was added to each Eppendorf and incubated for 5 minutes at 37ºC. Then 600 µL of culture medium was added for trypsin inactivation and the cellular suspension was recovered for the analysis and re-suspended in a clean medium. Cell cycle distribution was studied using the Cell Cycle Staining Kit (Beckman Coulter, USA) following the manufacturer's instructions in a Navios flow cytometer (Beckman Coulter, USA). Data analysis was by the ModFit LT Version 5.0 software (Verity Software House, USA).

2.5.4 Cell Adhesion evaluation

Cell morphology and location in the 3D environment was determined via an actin/DAPI staining of the samples after 1, 3 and 7 days of culture. The cultured samples were first washed twice with DPBS and the cells were fixed with neutral buffered formalin 10% solution (Sigma-Aldrich) for 15 minutes at RT, then washed twice with DPBS and permeabilized with Triton100X 0.1 vol% solution in DPBS for 5 minutes at RT. They

were then washed twice with DPBS and incubated with phalloidin-FITC conjugated (Invitrogen) (1:100) and DAPI (Sigma-Aldrich) (1:500) for 1 hour in darkness at RT with stirring. Finally, they were placed on glass slides with mounting medium and visualized through a DMI3000b fluorescence microscope (Leica Microsystems, Germany). The resulting images were assembled on ImageJ software (National Institutes of Health, USA).

2.5.5 Supernatant cellular staining

To confirm the presence of cells in the supernatants, before starting the actin/DAPI staining, the cell medium removed from 7 days samples was centrifuged (5 minutes, 250 G) and the cell pellet resuspended in 1:7000 Hoechst 33342 (Sigma Aldrich) solution in DPBS for DNA-specific staining. Samples were incubated for 30 minutes at RT in darkness and then visualized on glass slides with a DMI3000b fluorescence microscope (Leica Microsystems, Germany). The resulting images were assembled on ImageJ software (National Institutes of Health, USA).

3. Results and Discussion

3.1. Microsphere production, functionalization and characterizations

The microspheres were produced by an oil/water (o/w) emulsion using PVA as stabilizer from the two different previously synthesized biostable co-polymers (p(EA-EMA-0%AA) and p(EA-EMA-5% AA)). 5% AA microspheres without ferrite show a smooth surface with a spherical and homogeneous shape, while magnetic microspheres, which contain ferrite, show a rougher surface with some distortions due to the magnetic nanoparticles in their structure (**Figure 3**). In both cases, the microspheres are porous (**Figure 3b and 3e**) probably due to the solvent evaporation process during the o/w emulsion. The same morphology change pattern was described

by Vikingsson et al. [19] when producing magnetic PLLA (poly-L-lactic acid) microspheres via the same o/w emulsion method. Size distribution ranged between 17 and 287 µm (**Figure 3f**) with an average size of 143± 38 µm for the magnetic 5% AA microspheres. 5% microspheres without ferrite had a size distribution between 50 and 203 µm (**Figure 3c**) with an average size of 149 ± 31µm.

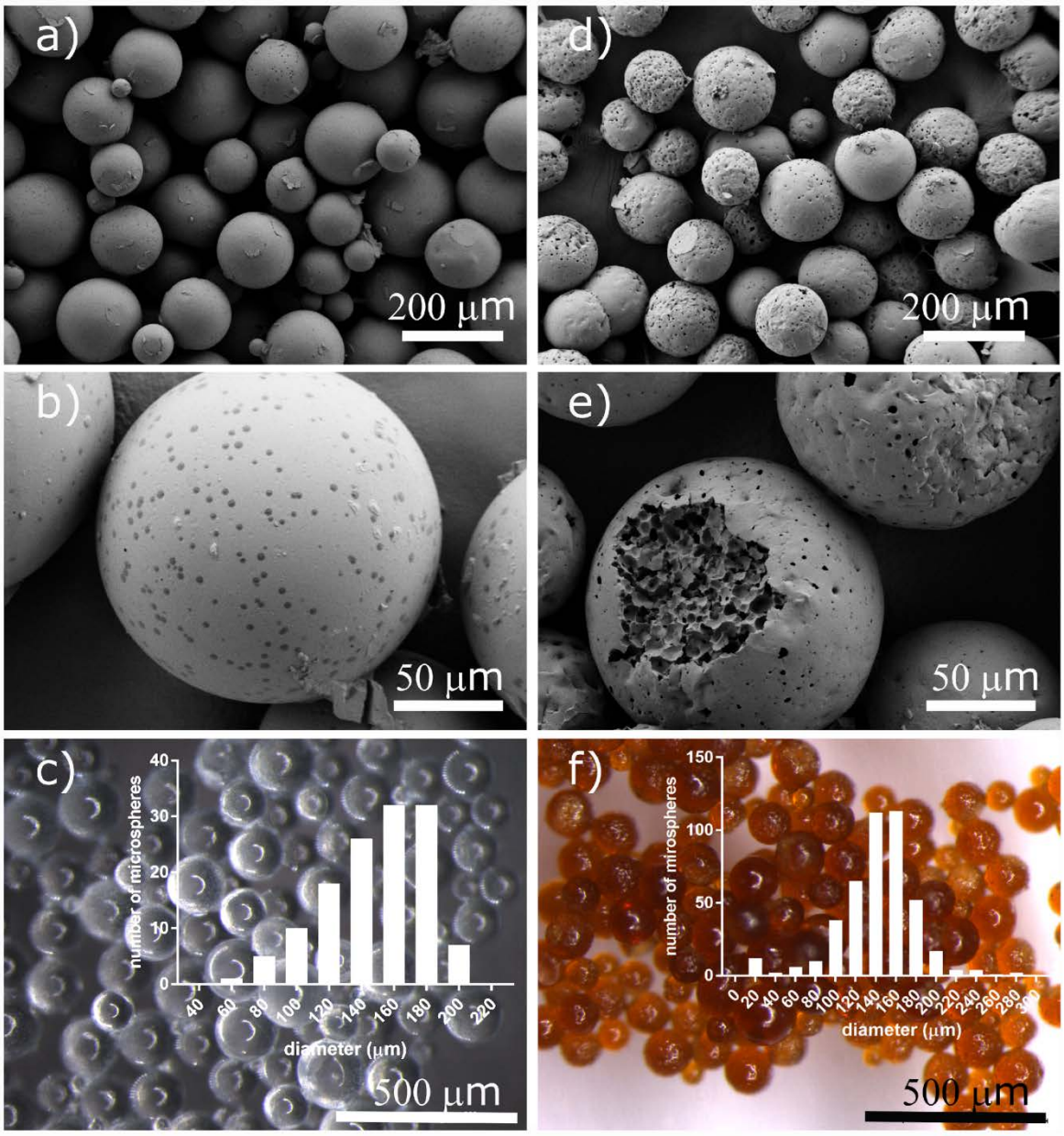

Figure 3. FESEM (field emission scanning electron microscope) images and size distribution of 5%AA (acrylic acid) microspheres. a,b,c) Microspheres without ferrite. d,e,f) Magnetic ferrite microspheres.

The microspheres from the 5% AA polymer were the only ones functionalized by HA covalent grafting, since this would require the presence of COOH groups on their

surface. Low molecular weight HA (~320000 Da) 5% w/v solution was used for functionalization and the toluidine blue indirect method showed that 87% of the HA used was covalently bound to the microsphere surface at the end of the process, which indicates good efficiency.[20] In the case of FN, this biomolecule was adsorbed to the surface of magnetic microspheres from both of the co-polymers produced (0% AA and 5% AA). By combining the SDS-PAGE and microBCA results it was possible to establish that in the case of 0 % AA microspheres the efficiency of the process (% FN adsorbed from the FN used in the functionalization) was 79% and 80% for the 5% AA microspheres.

3.2 Porcine mesenchymal stem cell culture

This study tested the behavior of pMSC in terms of cellular proliferation when grown in 3D environments. Three different 3D environments were evaluated: 0% AA magnetic microspheres with adsorbed FN, 5% AA magnetic microspheres with adsorbed FN and 5% AA magnetic microspheres with covalently bound HA, as well as a conventional bi-dimensional culture in TCPS.

When studying cell proliferation, the measurement of total DNA (PicoGreen™ commercial kit) showed that pMSCs proliferate at a constant rate regardless of the composition of the polymeric material in the FN-coated microspheres (**Figure 4a**). This rate of proliferation was initially lower than that of cells grown in conventional monolayers on TCPS plates, however after 3 days of culture the proliferation rate was similar in the 3D environments and 2D control (**Figure 4b**). After 7 days, 5% AA-FN microspheres showed a cell proliferation equivalent to that of the conventional 2D culture (**Figure 4b**). The HA coating did not allow good initial cell proliferation (**Figure 4a**), although after 7 days cell number had reached similar levels to those of the FN coated microspheres (**Figure 4b**).

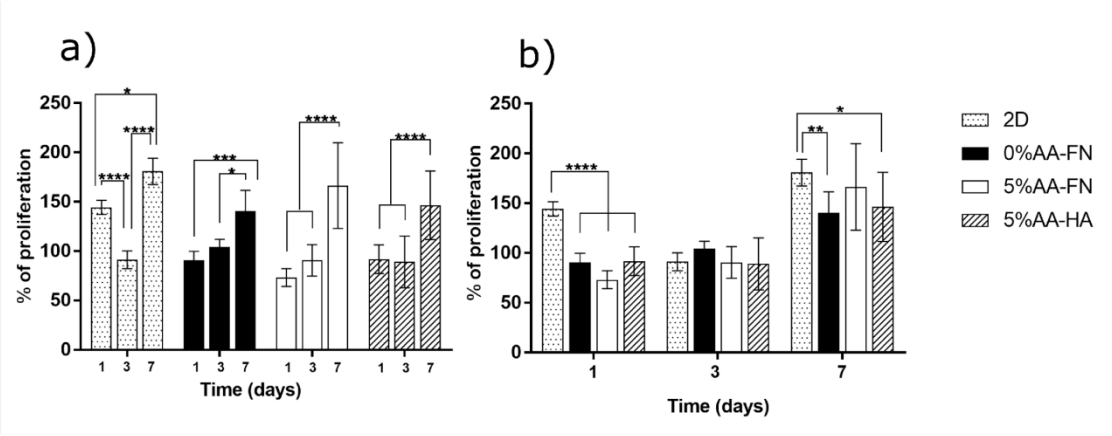

Figure 4. Results of dsDNA quantitation by PicoGreen™. Data was plotted twice, grouped according to cell culture environment (a) or time (b), to facilitate its interpretation. Results are expressed as % of proliferation (increase in cell density in reference to the initial cell density when harvesting the culture). The level of statistical significance is shown by the following legend: (*) p-value ≤ 0,05, (**) p-value ≤ 0,01, (***) p-value ≤ 0,001, (****) p-value ≤ 0,0001. AA: acrylic acid, FN: fibronectin, HA: hyaluronic acid.

The results of the cell cycle distribution studied by flow cytometry are given in **Figure 5**. The data show that in the 2D culture the number of cells facing an active proliferation process (phase S cells) starts with about half the cells at day 1 and decreases with time (**Figure 5b**), which is the normal pattern of a culture saturated by cellular confluence. This result reinforces the previous finding that initial proliferation was higher in 2D than 3D. However, in the case of the microspheres, the different environments showed a higher G0-G1 cellular fraction than in the 2D conventional culture (**Figure 5a**) (especially for 1 and 3 days), which means that pMSC grow at a lower proliferation rate in 3D environments. This finding is important as it shows the fate of pMSC can be controlled by this biomimetic culture platform. However, the most surprising finding was that after 7 days no cells were found directly adhered to the different microspheres. In the HA coated microspheres, even at 3 days, the number of cells detected by the cytometer (adhered to the microspheres surface and recovered after trypsinization and detected by the cytometer) was 1/5 of the cells attached to the

FN-coated microspheres. This suggests that the cell cycle data for 5%AA-HA microspheres on day 3 could be considered an outlier, as not enough cells were analyzed (**Figure 5**). As expected, HA hindered cell adhesion more than FN and consequently fewer cells could be recovered from the microsphere surface. Note that the fact of not detecting enough cells indicates that they were lost during sample manipulation, as shown in **Figure 7**. This suggests that the cells grew in the space between the microspheres and were lost in the analysis during the supernatant removal (**Figure7**).

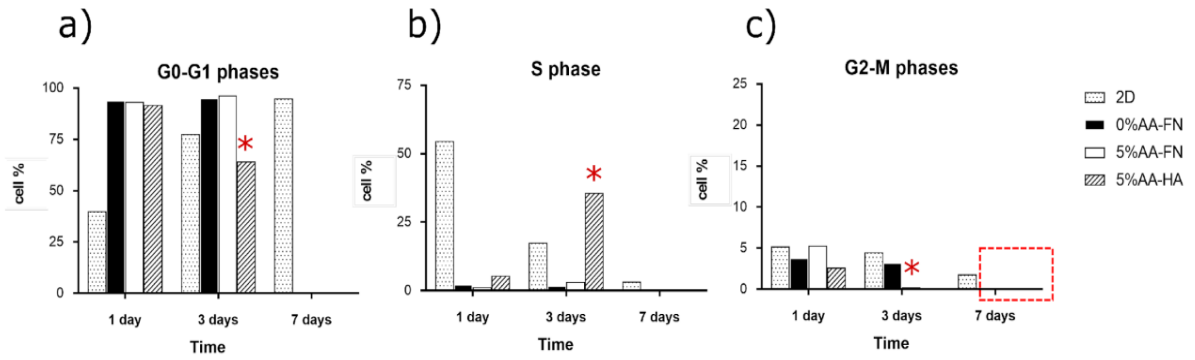

Figure 5. Results of cell cycle assessment by flow cytometry. (*) indicates that for these samples the number of cells analyzed was not enough for cell cycle fitting and so the data is not reliable. (---) indicates that no cells were detected. AA: acrylic acid, FN: fibronectin, HA: hyaluronic acid.

The actin/DAPI staining shows cell location and morphology after culture in the 3D environments evaluated. As seen in **Figure 6**, more cells are located on the microsphere surfaces in the FN-coated microspheres (**Figure 6e-g, i-k**) than in those functionalized by HA (**Figure 6m-o**), as could be expected, since FN coating promotes cell adhesion. The differences in the polymer composition between FN-coated microspheres seem to indicate no significant change in terms of cells adhered to the microspheres. Areas of high cellular density are formed on day 3. Again, after 7 days some samples have no cells on the microspheres surface (**Figure 6h, l, p**). However, when the assay was repeated with less manipulation of the samples during the

protocol, high cell density areas were found, especially in the FN-coated environments after 7 days (**Figure 6g,k,o**). In the case of dsDNA the assay was carried out in the Eppendorf culture tubes, which involves less sample manipulation, and this pattern was not detected.

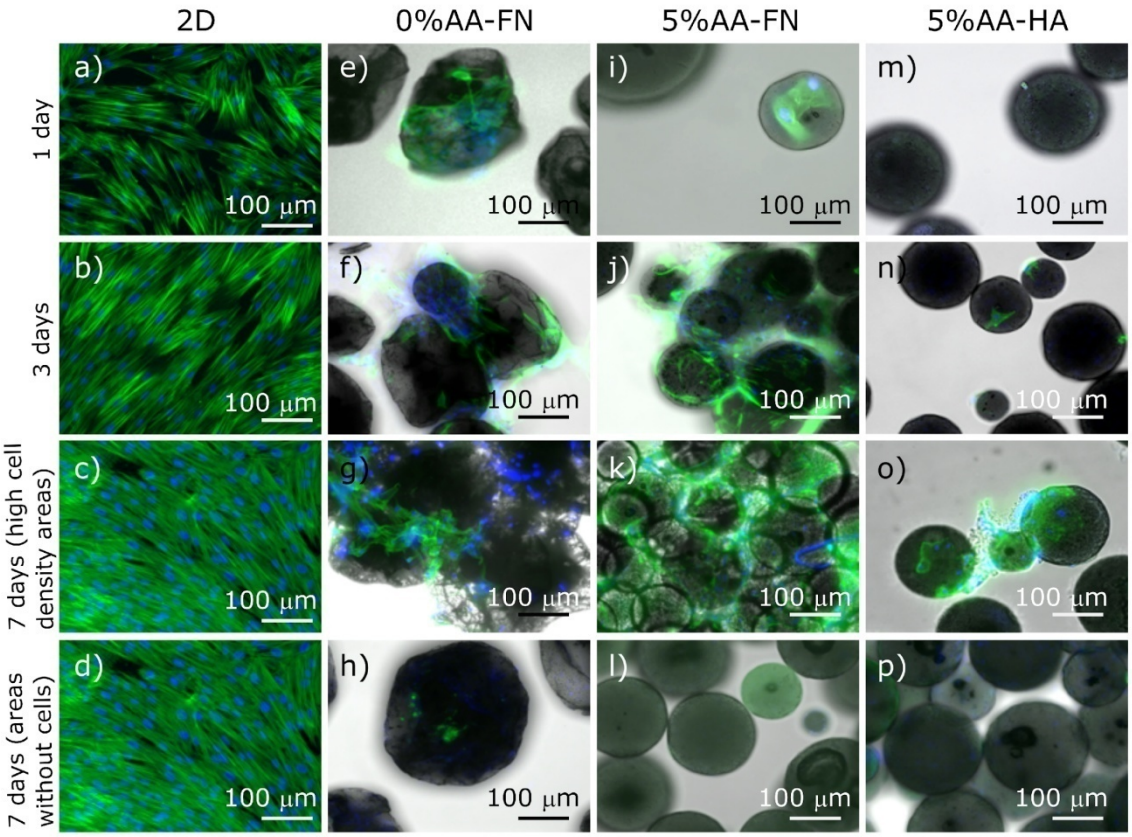

Figure 6. Images of the actin/DAPI staining. Cell nuclei appear in blue and actin cytoskeleton in green. There are 2 rows representing 7 days in order to show regions of higher cellular densities and regions without cells, both present in the analyzed samples of the 3D environments. AA: acrylic acid, FN: fibronectin, HA: hyaluronic acid.

Taken together with those of the cytometric measurements, these results suggest that in the different 3D environments the cells tend to proliferate forming aggregates not directly adhered to the microspheres. This means that when they produce their own ECM the cells are able to form 3D cellular aggregates filling the spaces among microspheres. In the techniques requiring greater handling of the sample (flow

cytometry and actin/DAPI staining) these aggregates were lost in one of the steps involving supernatant removal, as confirmed by analysis of the supernatant removed, which showed positive cell nuclei staining with Hoechst 33342 (**Figure 7d-f**). Levato et al.[9] studied the interplay between material surface properties, functionalization conditions and cell adhesion when biomaterials are surface modified with bioactive molecules. Working with molecules known to be promoters of cell adhesion and proliferation (collagen and RGD peptides) on PLA carriers, physisorption appears to be an unsuitable procedure to improve MSC response. After 5 days the cells reached confluence, whether covalently bound or physisorbed. However, the cell population then started to decrease in all the non-covalently coated samples, as in the present study. The smaller cell population at long culture times seems to be mostly because the physical interactions between the biomolecule and the surface of the microspheres are too weak to allow efficient grafting. The ECM remodeling caused by the cells triggers the separation of the coating, or at least of the cellular aggregates, and the ECM naturally produced from the material with culture time. Levato et al. also found formations of large cell-microcarrier aggregates in static culture conditions and that cell proliferation tended to occur in individual or small groups of microspheres, in agreement with the cellular distribution patterns of the actin/ DAPI staining in this work. [9] These patterns would probably be avoided in dynamic culture conditions.

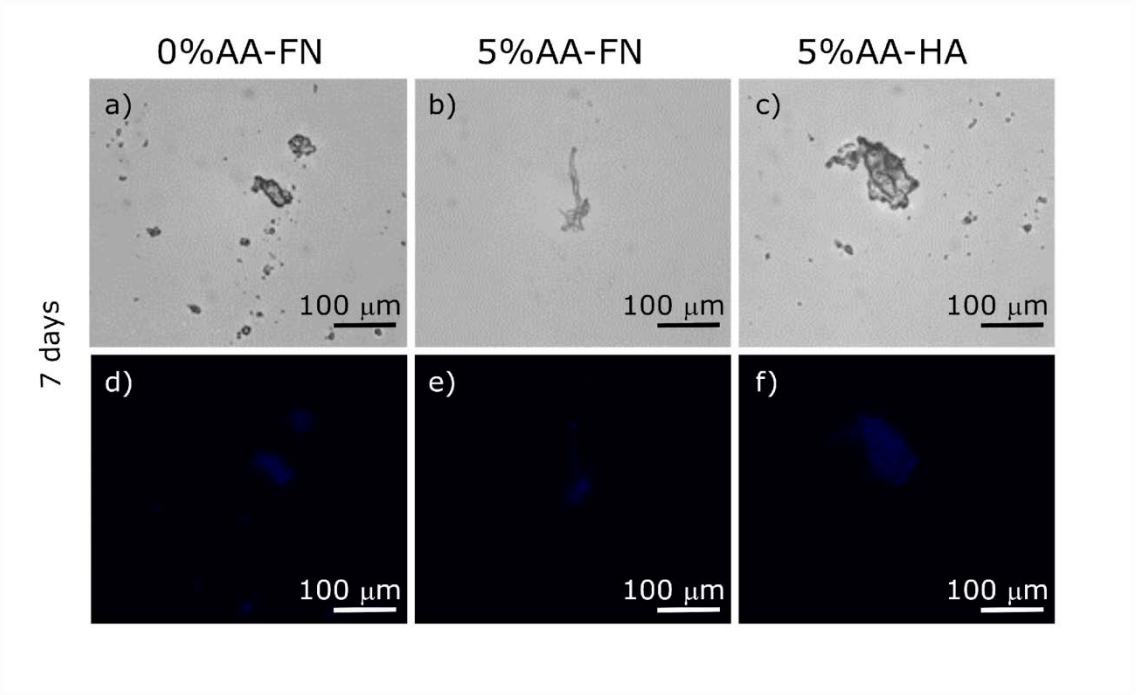

Figure 7. Supernatant cellular staining of actin/DAPI samples. Images of supernatants of cell media removed after 7 days of culture in different 3D environments. 1st row: bright field images. 2nd row: fluorescent nucleic acids staining with Hoechst 33342. AA: acrylic acid, FN: fibronectin, HA: hyaluronic acid.

4. Conclusions

This study shows that the chief advantage of the proposed cell culture system is that the microspheres constitute a more flexible and biomimetic 3D environment that can be freely remodeled and restructured by the cells and their natural ECM without the restrictions imposed by a rigid material.

In terms of cell proliferation, the environments based on 3D microspheres showed lower initial proliferation rates than the TCPS at day 1. However, cell proliferation was equivalent to the 2D conventional culture after 7 days, especially in the 5% AA-FN microspheres based environment. The cell cycle assessment supports these lower initial proliferation rates in the 3D environments, as it shows higher G0-G1 cellular fractions for the microspheres based environments than the 2D culture. Finally, the

actin/DAPI staining showed how cells grow in biomimetic microenvironments, forming larger cellular aggregates and that the cells surround the microspheres when a fibronectin coating is applied to their surface, while there was hardly any cell adhesion to the microspheres coated with hyaluronic acid. However, the tests described here are simply a proof-of-concept to show that the proposed environment carries out cell adhesion and proliferation of pMSC in biosimilar conditions and promises to be a useful cell culture tool whose possibilities deserve further exploration. Future experiments will allow us to investigate the possibilities of this environment to manipulate the stem cell fate and guide their differentiation.

Finally, thanks to the microspheres' magnetic properties, after the culture it was possible to analyze both cells and the support and to recover the cells separately without the need for an aggressive procedure by simple trypsinization of the sample. This shows it to be a versatile system in terms of handling samples and that it could be used to perform a wide variety of different cell analysis techniques, besides promising to be a cell culture tool with good potential in the field of tissue engineering and cell therapy.

Abbreviations

AA, acrylic acid; BSA, bovine serum albumin; DMEM, Dulbecco's modified eagle medium; DPBS, Dulbecco's phosphate-buffered saline; ECM, extracellular matrix; EDC, 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride; EMA, ethyl methacrylate; FBS, fetal bovine serum; P/S, penicillin/streptomycin; FESEM, field emission scanning electron microscopy; FN, fibronectin; GFs, growth factors; GvHD, graft versus host disease; HA, hyaluronic acid; p(EA)poly(ethyl acrylate); HSC, hematopoietic stem cells; MNPs, magnetic nanoparticles; MSC, mesenchymal stem cells; NHS, N-hydroxysuccinimide; PEG-di-NH_2, poly (ethylene glycol)bis(3-aminipropyl)terminated; pMSC, porcine mesenchymal stem cell; PVA, polyvinyl alcohol; RT, room temperature; TB, toluidine blue; TCPS, tissue culture polystyrene.

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