Bioinstructive microparticles for self-assembly of mesenchymal stem Cell-3D tumor spheroids

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1. Introduction

Presently, in vitro preclinical validation platforms recommended by regulatory agencies to screen for candidate anti-cancer therapeutics are mainly based on the use of 2D cell monolayers and in vivo animal models [1,2]. Both of these are increasingly seen as inadequate approximations of the complex human tumor microenvironment (TME) [3,4]. 2D monocultures are unable to correctly recapitulate spatial organization, cell-cell and cell-matrix interactions, biochemical cues and TME heterogeneity [5]. While animal models are recognizably expensive, laborious and ethically controversial alternatives, often lacking correct representation of the human tumor stroma or tumor infiltrating immune system [6,7]. Improved representation of the TME in in vitro tumor mimicking platforms is urgent for improving basic cancer biology research (e.g., new biomarkers and biological targets) and drug-screening procedures.

Three-dimensional multicellular tumor spheroids (3D-MCTS) currently receive an increased attention in the field of anti-cancer drug discovery due to their ability to robustly recapitulate specific features of in vivo tumors [8]. In fact, such models allow a straightforward cellular stroma representation by combination of malignant and non-malignant cell populations in a 3D environment that promotes cell-cell contacts and communication [9]. Cells cultured in 3D self-aggregate to form in vitro microtumors that mimic oxygen/nutrient and pH gradients of human solid tumors [10]. Such provides an in vivo surrogate environment in which cancer cells phenotypical, genetical and metabolic heterogeneity can be easily recapitulated. However, despite better portraying the diverse cellular components of tumor microenvironment (TME), in general 3D-MCTS lack pre-existing extracellular matrix (ECM) components, with ECM being deposited by cells during culture [11]. Inclusion of ECM-mimetics (e.g., hyaluronic acid [12], collagen [13], fibrinogen [14]), may improve 3D-MCTS ability to correctly

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mimic tumor specific-ECM at an early stage. This supportive matrix is known to influence the process of metastasis, invasion, and acquisition of multi-drug resistance [15].

Regarding lung cancer ECM, one key component that has been associated with poor patient prognosis is Hyaluronic acid (HyA) [16-18]. This biopolymer is comprised by disaccharides of α-glucuronic acid and N-acetyl-d-glucosamine is found ubiquitously in human tissues [19,20]. Several studies observed that cancer cells associated to a HyA-rich ECM environment exhibit an increased expression of two key cellular receptors: (i) CD44, a marker associated with multidrug resistance [21,22]; and (ii) RHAMM (receptor for hyaluronan-mediated motility, also known as cluster of differentiation 168 – CD168), a receptor associated with invasion and metastasis through changes in motility, polarity and directed migration, as well as involved in matrix remodeling proteins production (e.g., MMP-9 or PAI-1) [20]. Apart from this, also the cellular components of the TME must be included in the design of 3D in vitro tumor models so as fully recapitulate malignant-stromal cells heterogeneity.

In this context, human mesenchymal stem cells (MSCs) have been recently recognized as important cellular constituents of the TME playing paramount roles in multiple cancers [23–26]. MSCs have been involved in the: (i) regulation of the immune response [27,28], (ii) modification of the surrounding tumor-ECM [29,30], (iii) conversion of fibroblasts to CAFs [31], (iv) initiation of epithelial-to-mesenchymal transition (EMT) by cancer cells [32], and (v) metastasis [27]. Despite these contributions to cancer progression, some reports also emphasize MSCs role in inhibiting and suppressing tumor proliferation, invasion and metastasis [33]. The different influences of MSCs in lung cancer have been recently revised elsewhere [30]. Given their polyvalent activity, the representation of MSCs in 3D models is critical for the correct recapitulation of human tumors in an in vitro setting. However, to date, few works have explored MSCs inclusion in an ECM-containing 3D environment, and even fewer in a heterotypic model that includes the main populations of the TME (i.e., cancer cells and fibroblasts) [34].

From this standpoint, herein we designed a multicomponent 3D in vitro lung tumor model that simultaneously recapitulates TME cellular heterogeneity and cell-ECM crosstalk. The latter was accomplished by incorporation of poly-(ε-caprolactone) microparticles (MPs) coated with a bioinstructive HyA multilayer produced via the layer-by-layer (LbL) deposition technique. Moreover, hBM-MSCs were also co-cultured with A549 cancer cells and fibroblasts for the first time in 3D to evaluate their influence in the response to chemotherapeutics. Overall the obtained results demonstrate that bioinstructive MPs promote the assembly of robust and reproducible 3D-MCTS and that the inclusion of hBM-MSCs must be considered in the design of in vivo mimicking 3D tumor models were the infiltration of these cells occurs (e.g., lung, breast, prostate, colorectal).

2. Materials and methods

2.1. Materials

Polycaprolactone (PCL; Mn: 80 000 Da), Polyvinyl Alcohol (PVA; MW: 30 000–70 000 Da) and Poly-ε-lysine hydrobromide (PLL; MW: 30 000–70 000 Da) were acquired from Laborspirit (Loures, Portugal). Chitosan chloride (MW: 50 000–150 000 Da) was acquired from Novamatrix (Sandvika, Norway). Hyaluronic acid sodium salt polymer (MW: 80 000–100 000 Da) was purchased from Carbosynth Limited (Berksire, United Kingdom). Ultra-Low-Adhesion (ULA) round-bottom 96-wells plates, Fetal Bovine Serum (FBS; E.U. approved, South America origin), Dulbecco’s Modified Eagle Medium-High Glucose (DMEM-HG), phosphate buffered saline, pH = 7.4 without Ca²⁺ and Mg²⁺ (dPBS), Ham’s F-12K Kaign’s Medium (HAMs-F12), Alpha Modified Eagle’s Medium (α-MEM), TrypLE™ Express, Goat anti-Mouse IgG (H + L) Alexa Fluor 488 (Alexa 488), Secondary Antibody, anti-human Collagen I Antibody (SD8-G9), anti-human E-cadherin-PE antibody, Calcein-AM, Propidium Iodide (PI) were all purchased from Thermofisher Scientific Inc (Alfagene, Portugal). Anti-human CD44-FITC was purchased from Taper (Grupo Taper S.A., Lisboa, Portugal). All other reagents and salts were of analytical grade and used without further purification.

2.2. Methods

2.2.1. PCL microparticles production

PCL microparticles where produced by using the oil-in-water (O1/W1) emulsion-solvent evaporation technique. The oil phase (O1) was comprised by a 5% (w/v) PCL solution. The aqueous phase (W1) consisted of 0.5% (w/v) PVA aqueous solution. To form the water-oil emulsion 8 mL of PCL (O1 phase) were dispersed into 150 mL of PVA by using a high precision piezoelectric-based air pumping system (OBI MK3 –Microfluidic Flow Control System, Elveflow™, France), operated at an air pressure of 5 bar. The microparticle-containing solution was then placed under horizontal stirring at 170 rpm during at least 8 h, at room temperature (RT), inside a fume-hood. The recovered PCL microparticles were washed 3 times with deionize water by using re-suspension/centrifugation cycles (1000 rpm, 5 min). The particles were then sieved through stainless steel sieves to obtain particle size ranges from 63 μm to 100 μm. All particles were freeze dried for 48 h and stored in a moisture free atmosphere until further use.

2.2.2. Bioinstructive microparticles production via layer-by-layer

Prior to biopolymers surface functionalization microparticles were subjected to plasma treatment. For this purpose, 200 mg of sieved microparticles where placed in a sterile beaker and subjected to plasma treatment by using atmospheric air charged at 30 V, for 5 min, RT. For Layer-by-layer (LbL) surface functionalization 250 mg of plasma treated PCL MPs (LbL-MPs, negative charge), were then immersed in 20 mL of PLL (1 mg/mL, 0.22 μm filtered), for 10 min, and washed in distilled water for 5 min. For the buildup of the negative layer, PLL-MPs were transferred into an HyA solution (1 mg/mL, filtered 0.22 μm) for 10 min, and re-washed for 5 min. This process was repeated 3 times to allow the formation of 3 PLL/HyA bilayers.

2.2.3. Zeta potential analysis of polyelectrolyte polymers deposition

Zeta potential measurements were used to verify the buildup of polyelectrolyte layers in MPs surface during LbL. This analysis was also performed in plasma treated, non-coated, PCL MPs that were used as control. All measurements where performed in a ZetaSizer Nano ZS (Malvern, Worcestershire, UK), at 25 °C by using the automatic mode and a disposable cell (DTS1070). The ZetaSizer software was used to record and convert the electrophoretic mobility data to zeta potential (v. 7.04).

2.2.4. Microparticles characterization

Microparticles morphology and particle size was evaluated by optical contrast light microscopy (Primostar, Carl Zeiss, Germany). The acquired images where analyzed using open-source software ImageJ v1.08 (NIH, Bethesda, ML) [35] and microparticles size distribution was determined via a supervised algorithm that analyzed a minimum of 300 microparticles. Scanning Electron microscopy (SEM) imaging was used to analyze microparticle morphology and surface topography. For SEM analysis, were dispersed in deionized water and drop-wise added to an aluminum stub containing a glued tissue culture treated polystyrene insert (TCP5, Starstedt, Lisbon, Portugal). The samples were then dried at 37 °C overnight, sputter coated with gold/palladium and observed in a Hitachi S-4100 scanning electron microscope (Hitachi, Japan) operated at a voltage in the range of 15–25 kV and at various magnifications.

2.2.5. Cell culture

All cells were manipulated in aseptic conditions and cultivated at
37 °C in a humidified temperature-controlled incubator with a 5% CO₂ atmosphere. The non-small cell lung carcinoma cell line A549 (ATCC CRM-CCL-185™) was cultured in HAMs-F12 medium supplemented with 10% (v/v) FBS and 1% (v/v) ATB. The human primary dermal fibroblasts cell line (HF) (ATCC®-PCS-201-012™) was cultured in DMEM-HG supplemented with 10% (v/v) FBS and 1% (v/v) ATB. Human adult Bone Marrow-derived Mesenchymal Stem Cells (hBM-MSCs) (ATCC®-PCS-500-012™) were cultured in α-MEM supplemented with 10% (v/v) FBS and 1% (v/v) ATB. All cells where detached from culture flasks upon attaining approximately 80–85% confluency by using TrypLE™ Express Enzyme (1×) detaching reagent. Throughout all studies hBM-MSCs were used from passage 3 to 7 to assure trilineage cell ratios (Table 1), by using the liquid-overlay technique, also known as forced-folding methodology. To form 3D-MCTS cells were initially multiple or single-cell suspensions comprised by the different cell populations were cultured in ULA round bottom 96-wells plates. For 3D-MCTS hybrid spheroids cells were cultured with Lbl-MPs. Prior to each assay, Lbl-MPs were sterilized under UV light (30 min) and re-suspended in complete HAMs-F12 cell culture medium. To produce different 3D in vitro models that would better recapitulate tumor microenvironment cellular heterogeneity, different parameters were manipulated, namely: (i) the cell number per well, (ii) the cell-to-cell ratios between malignant and mesenchymal/endothelial cells and (iii) the cell-to-particle ratios. The specific ratios used for each condition are summarized in (Table 1).

### 2.2.6. 3D In vitro lung tumor models assembly via liquid-overlay technique

Homotypic monoculture 3D-MCTS formed by A549 cells, heterotypic co-culture spheroids A549:HF and A549:hBM-MSCs cells, or tri-cultures: A549:HF:hBM-MSCs cells, were self-aggregated at different cell ratios (Table 1), by using the liquid-overlay technique, also known as forced-folding methodology. To form 3D-MCTS cells were initially multiple or single-cell suspensions comprised by the different cell populations were cultured in ULA round bottom 96-wells plates. For 3D-MCTS hybrid spheroids cells were cultured with Lbl-MPs. Prior to each assay, Lbl-MPs were sterilized under UV light (30 min) and re-suspended in complete HAMs-F12 cell culture medium. To produce different 3D in vitro models that would better recapitulate tumor microenvironment cellular heterogeneity, different parameters were manipulated, namely: (i) the cell number per well, (ii) the cell-to-cell ratios between malignant and mesenchymal/endothelial cells and (iii) the cell-to-particle ratios. The specific ratios used for each condition are summarized in (Table 1).

### 2.2.7. 3D tumor microtissues characterization

3D in vitro hybrid spheroids morphology, growth and circularity were analyzed overtime via optical contrast microscopy by using an inverted microscope (Primovert, Carl Zeiss, Germany). Micrographs were acquired at specific time points (day 1, 7 and 14), and a minimum of 6 spheroids were analyzed per condition/time point. Image analysis was carried out by using the open-source software ImageJ (Fiji package) and a supervised algorithm based on the code developed by Ivanov and co-workers [43]. 3D in vitro tumor models' circularity was evaluated in ImageJ v1.08 (NIH, Bethesda, MD), as previously described in the literature [44]. Circularity was calculated from optical contrast microscopy images by using the following equation:

\[
\text{Circularity} = 4\pi \left(\frac{\text{area}}{\text{perimeter}^2}\right)
\]

For SEM analysis, 3D spheroids were processed as reported in the literature [37,45,46]. In brief, 3D-MCTS with and without Lbl-MPs were removed from culture media and washed with dPBS, fixed in formaldehyde 4% (v/v, in dPBS), at 37 °C for 2 h. The samples were then subjected to dehydration with graded ethanol concentrations (25%, 50%, 75%, 90% (v/v)), for 20 min. All samples were then carefully mounted in aluminum stubs by using double sided adhesive carbon tape (Agar Scientific, Essex, United Kingdom) and sputter coated with gold/palladium. All spheroids were imaged in a scanning electron microscope (Hitachi S-4100), operated at different voltages and various magnifications.

### 2.2.8. Cell viability assays

The cell viability and necrotic core formation of different homotypic and heterotypic in vitro 3D microtumors (3D-MCTSs and 3D-MCTS Lbl-MPs), were analyzed at specific time points (7 and 14 days) by using the Alamar Blue® Cell Viability Assay and Calcein-AM/PI, live/dead assay. Alamar blue was used to access 3D tumor model’s viability during the initial steps of homotypic and heterotypic mono-, dual and tri-co-cultures optimization. Both assays were performed in accordance with manufacturer instructions. Alamar Blue (resazurin active compound), reduction to resorufin was determined by fluorescence measurements \(\lambda_{ex}: 540 \text{ nm}, \lambda_{em}: 600 \text{ nm}\). Fluorescence data was recorded in a Synergy HTX microplate reader by using a 96-well black-clear bottom plate.

### 2.2.9. 3D-MCTS characterization by widefield and confocal laser scanning microscopy

The production of Collagen I was analyzed at specific time points (7 and 14 days) through immunocytochemistry. For this purpose, 3D-MCTSs were fixed in 4% formaldehyde (v/v, in dPBS), overnight, at RT. The spheroids were then carefully transferred to a new 96 well ULA plate, washed and incubated for 1 h with blocking solution (1% BSA in dPBS), at 4 °C, followed by a washing step with dPBS. Then 3D-MCTSs were incubated with E-cadherin-PE (5 μL/mL), or Collagen I (2.5 μL/mL) antibody overnight, at 4 °C, washed with dPBS for 3 times. Collagen I-stained spheroids where then incubated with Alexa488 secondary antibody for 1 h, RT. Acquistion of fluorescence micrographs was performed in an upright widefield microscope (Axio Imager M2, Carl Zeiss, Germany), or in laser scanning confocal microscopes (LSM 510 Meta, and LSM 880 Airyscan, Carl Zeiss, Germany). The analysis of acquired images was performed in Zeiss Zen Blue software (2017). To evaluate 3D-MCTS necrotic core formation, the spheroids were labelled with Calcein-AM (Cal-AM) (4 μg/mL) and Propidium Iodide (PI) (10 μg/mL) for 30 min at 37 °C, according to literature reports for 3D models [37]. Following incubation, the different 3D tumor models (3D-MCTSs and 3D-MCTS Lbl-MPs) were washed 3 times with dPBS and imaged immediately by fluorescence microscopy.

### 2.2.10. Histological analysis

Histological analysis of dual co-culture (A549:HF) and tri-coculture (A549:HF:hBM-MSCs 3D-MCTSs cultured in Lbl-MPs) was performed in order to analyze microtumors internal organization and collagen deposition. In brief, 3D-MCTSs with and without Lbl-MPs were removed from culture media and washed with dPBS, fixed in formaldehyde 4% (v/v, in dPBS), at 37 °C for 2 h. The samples were then subjected to...
dehydrating with growing concentrations of ethanol (25%, 50%, 75%, (v/v)), for 20 min, per condition. For histological analysis paraffin blocks containing 3D-MCTS were sliced into 5 μm thick samples and stained with: (i) Hematoxylin and Eosin (H&E), and (ii) Masson’s Trichrome for evaluation of collagen deposition. Histology slides were then analyzed by using an inverted optical contrast light microscope (Primovert, Carl Zeiss, Germany) equipped with a 3Mpix color camera (Zeiss 105, Carl Zeiss, Germany). All images were acquired and processed in Zeiss Zen Blue Software (2017).

2.2.11. Flow cytometry analysis
The effect of hyaluronan bioinstructive LbL-MPs in 3D-MCTS CD44 expression was analyzed by flow cytometry. For this analysis, 3D-MCTS and 3D-MCTS LbL-MPs with 7 days of culture were dissociated by incubation in a 1:1 mixture of Accumax® and TrypLE® Express for 30 min, at 37 °C. After this incubation period, spheroids were fully disrupted by gentle pipetting and incubated in HAMs-F12/10% FBS/1% ATB for 30 min and washed with dPBS for 3 times. The resulting single cells suspensions was then recovered by centrifugation. The different samples were incubated with anti-human CD44-FITC conjugate antibody (5 μL/mL in PBS) for 30 min, at 37 °C. Single cells were then recovered by centrifugation and washed with dPBS for 3 times. Prior to FCM analysis all samples were filtered through cell strainers (40 μm) to remove unbound particles. In addition, 2D monocultures of A549, HF and MSCs were also analyzed for their CD44 expression. Flow cytometry analysis all samples were filtered through cell strainers (40 μm) to remove unbound particles. In addition, 2D monocultures of A549, HF and MSCs were also analyzed for their CD44 expression. Flow cytometry analysis was carried out in a BD Accuri C6 flow cytometer (BD Bioscience, San Diego, CA, USA) were a total of 5 × 10^5 events per sample were acquired in the ROI of interest. Flow cytometry data was processed and analyzed in FCS Express software (v. 6.0, trial license).

2.2.12. Chemotherapeutic drug cytotoxicity screening in 3D-MCTS platforms
Obtained 3D-MCTS were cultured for 7 days on HAMs-F12 medium to be used as testing platforms for Doxorubicin cytotoxicity evaluation in monotypic monocultures (A549 cells), heterotypic co-cultures (A549:HF), and triple co-cultures (A549:HF:hBM-MSCs). Upon achieving the desired culture time, 3D-MCTS were incubated with Doxorubicin (Dox) chemotherapeutic agent at different concentrations ranging from 0.7 to 17 μM, over a period of 72 h. Cellular viability of 3D-MCTS was then evaluated by using a luminescence-based assay specifically designed to quantify cellular ATP in in vitro 3D cellular aggregates assembled either via scaffold-based or scaffold free methods (Cell Titer Glo™ Luminescent cell viability assay, Promega, Madison, WI, USA). CellTiter-Glo® assays were performed according to the manufacturer instructions. In brief, following incubation with Dox the medium was removed and 3D-MCTS were incubated with a mixture of HAMs-F12/10 %FBS/1% ATB medium and CellTiter-Glo® reagent at a 1:1 ratio. The samples were incubated for 25 min, at RT. Luminescence was then measured in 96-well flat-bottom opaque white plates by using a Synergy HTX microplate reader. Non-treated 3D-MCTS were used as controls.

2.2.13. Statistical analysis
All statistical analysis was performed using Graphpad Prism 6 Software (Prism 6™). One-way analysis of variance (One-ANOVA) and Two-way analysis of variance (Two-ANOVA) with Holm-Sidak’s post-hoc test. A minimum of 6 replicates was used for statistical analysis. Unless otherwise indicated, p < 0.05 was considered statistically significant.

3. Results and discussion
The establishment of in vitro 3D microtumor models relevant for drug screening at preclinical discovery stages must emulate tumor cellular heterogeneity and biomacromolecular microenvironment, so as to provide a highly robust in vitro/in vivo correlation [47]. Currently, the great majority of 3D tumor models for in vitro drug screening are based on the use of scaffold-free cell-agglomerates, i.e., 3D spheroids. However, by definition, these models lack pre-existent tumor-ECM components, being a rather simplistic representation of the complex tumor microenvironment (TME) [8]. One approach to overcome such limitations is the inclusion of ECM-like scaffolds based on micro- particles functionalized with bioinstructive tumor-ECM components (e.g., collagen, hyaluronan, etc) [48]. Microparticle technology has been extensively used in the field of tissue engineering due to their ability to support cell attachment, proliferation and promote a robust self-aggregation [49]. MPs co-cultured with different types of cells can also incorporate mechanical, biochemical and biomolecular tissue-specific cues.

As recently reviewed [50], few reports have explored microparticles as a strategy to include tumor-ECM mimetic components into spherical in vitro 3D tumor models. In general, microsphere-based 3D tumor models employ synthetic, non-bioinstructive microparticles. In this work LbL-MPs (63–100 μm) were used as a cost-effective and straightforward strategy to integrate virtually any ECM-mimetic substrates into in vitro assembled 3D-MCTS by using the LbL functionalization technology.

In the context of bioengineered in vitro tumor models, spherically-structured polymeric microparticles provide a unique surface for prompting rapid cells adhesion/aggregation, proliferation and for functionalization with tumor-ECM mimics. Taking advantage of this potential, we have developed a microparticle-based platform coated, via layer-by-layer (LbL), with a positive poly amino acid (Poly-l-Lysine) and negatively charged hyaluronan (HyA), a key component of tumor-ECM that provides adhesion hotspots to cells and also triggers important biochemical (Fig. 1). These so-called bioinstructive microparticles (LbL-MPs) were then used as scaffolding platforms establish a unique tri-coculture model comprising A549 lung cancer cells, human mesenchymal stem cells attached to bioinstructive, HyA-coated microparticles.
fibroblasts and bone marrow-derived mesenchymal stem cells (hBM-MSCs) (Fig. 1). The proposed multifactorial approach of this study aims to recapitulate the malignant-stromal cellular heterogeneity and provide a robust representation of the tumor microenvironment constituents. Up-to-date, very few studies have focused on the inclusion of MSCs in 3D *in vitro* tumor models [34]. As we recently reviewed, MSCs are an important element of *in vivo* human tumors, having diverse modulatory effects in tumors response to therapy, and should also be emulated *in vitro* [34]. Moreover, to the best of our knowledge, the present study is the first that includes stromal fibroblasts and mesenchymal stem cells in a 3D *in vitro* tumoral setting.

### 3.1. Microparticles production and layer-by-layer functionalization

Initially, the bioinstructive PCL-based MPs that served as structural platforms for 3D cells culture were formulated through the emulsion/solvent evaporation technique. The parameters influencing MPs production (polymer and surfactant concentration, oil-water solvent ratios, stirring), were optimized for efficient particles manufacture within the size range of 60–100 μm and with spherical morphology (Figure S1 and...
S2). Such sizes are significantly larger than those of malignant cells, allowing for adhesion of multiple cells to MPs, and the establishment of cell-cell interactions that are crucial for 3D aggregates self-assembly [49]. Following this preliminary size optimization, MPs were sieved and the percentage of particles in the desired size significantly increased from 49.5%, up to 95.2%. With this approach, the final MPs formulations exhibited a mean size of 83 μm and a relatively small coefficient of variation (CV: 14.86%) (Fig. 2B).

For PCL MPs surface functionalization with HyA ECM-mimetic coatings, the formulations were initially subjected to plasma treatment to increase hydrophilicity and promote a more efficient layer-by-layer (LbL) deposition of positively charged Poly-l-lysine (PLL) onto PCL particles surface. HyA was chosen to functionalize MPs outermost layer since this is a key component of human tumors-ECM, being generally associated to poor disease prognosis in patients with lung cancer [17,51]. Different reports in the literature support the use of PLL/HyA LbL layered particles for promoting embryonic stem cells adhesion/proliferation in 3D culture [52].

To study the surface deposition of polyelectrolyte polymer nanolayers and sequential surface charge reversal, zeta potential measurements were performed. The obtained negative zeta potential was in accordance with the charge of the HyA outermost polymer layer deposited onto MPs surface (Fig. 2C). Despite PLL/HyA 2 bilayers build up present negative zeta potential these films could be highly irregular. Hence, a coating of PLL/HyA 3 bilayers was selected to assure higher film homogeneity, whilst providing a rough surface for cells adhesion. Following LbL surface functionalization, PCL particles maintained both their size distribution, and morphology (Fig. 2, D1-3), with only a minor increase on surface roughness being observed (Fig. 2, D4-6). Furthermore, particles subjected to LbL functionalization with 3 bilayers of PLL/HyA showed no tendency to aggregate when incubated in culture medium (Supplementary Figure S3). Therefore, following the successful optimization of the LbL process for the obtention of biostructic microparticles coated with HyA, different assays were performed to access how the inclusion of LbL-MPs promoted 3D spheroids self-assembly and tumor mimicking features during in vitro culture.

3.2. 3D-MCTS assembly and morphological characterization

Culturing cells in vitro in a 3D setting, rather than in flat 2D substrates, provides the representation of key solid tumors hallmarks such as the formation of a necrotic core and the recapitulation of cell-cell tridimensional physical interactions and intercellular crosstalk [47,53–55]. With the objective of mimicking such characteristics, microparticle-based tri-coculture 3D tumor models containing non-small cell lung cancer cells (A549), human dermal fibroblasts (HF), and bone-marrow derived human mesenchymal stem cells (hBM-MSCs), were established by using disease relevant cell-cell ratios based on previous literature reports [30,36–38,41,42].

Prior to assembly of these complex models, a preliminary optimization of spheroid cultures involving mono and dual co-culture spheroids of A549:HF spheroids, the formation of tri-coculture spheroids at a ratio of 10:20:1 (A549:HF:hBM-MSCs) was investigated. The ratio of cancer cells to hBM-MSCs was selected from a previous report by Liu and co-workers, which discovered that smaller ratios of MSCs to A549 cells seem to favor pro-tumorigenic interactions in vitro [30]. This low ratio is also representative of what occurs in human tumors, since only a limited number of MSCs migrate from the bone marrow to the tumor mass in vivo [34].

Spheroids circularity and area analysis in the conditions where A549 cells were combined with hBM-MSCs, revealed that tri-co-culture spheroids had the smallest sizes and formed the most densely packed microtissues of all conditions including those comprising HyA MPs, as shown in Fig. 3.

3.3. 3D-MCTS cellular viability and necrotic core formation

Analysis of mono, dual and tri-co-culture 3D tumor microtissues, assembled by cells self-aggregation or guided by LbL-MPs anchoring platforms, demonstrated that sizes higher than 500 μm were easily obtained, indicating that the followed methodology is suitable to obtain large tumor spheres. As mentioned, in this size range, cells are subjected to nutrient/oxygen/pH gradients and generally start to form a characteristic necrotic core similar to that obtained in human solid tumors [58]. To analyze if a dense mass of necrotic cells was formed Live/Dead assays based on spheroids staining with Calcein-AM (Cal-
Fig. 3. 3D microtumors formed by Tri-coculture of A549:HF:hBM-MSCs and their evolution from 1 to 14 days of culture. (A) Schematics of 3D tri-coculture models established in 96 well ultra-low adhesion plates, at specific cancer-stromal cells ratios. (B) Spheroids area quantification, and (C) quantitative circularity measurements (1 = perfect circle, 0 = line) were performed by using image Analyze/Measure algorithms contained in ImageJ v.1.8.0. (D) 3D microtumors morphological analysis by optical contrast microscopy. Scale bar represents 500 μm.
AM)/PI were performed. As evidenced by fluorescence microscopy, in A549 MCTS containing or not LbL-MPs necrotic formation core was visible after 14 days of culture (Figure S10). Interestingly, in A549:HF LbL-MPs dual coculture 3D spheroids the necrotic core was readily visible at 7 days of culture, thus evidencing that HF stromal cells promote a faster establishment of the necrotic core. Moreover, in monoculture MCTS A549 containing 30 000 cells and LbL-MPs an increased number of dead cells was visible already at day 7, with clear necrotic core formation occurring at day 14 (Supplementary Figure S13). This indicates that LbL-MPs inclusion further approached these models to those obtained through co-culture with either HF or hBM-MSCs, possibly by physically decreasing penetration of nutrients and oxygen into the inner spheroid regions, or through HyA derived upregulation of adhesion mechanisms, as evidenced by CD44 upregulation (Fig. 9). Moreover, in all tested conditions the established necrotic core was also denser at 14 days, an important feature that influences the penetration of nutrients/oxygen into deep microtumors regions [59,60].

Live/dead analysis of tumor-mimicking tri-coculture (A549:HF:hBM-MSCs) 3D spheroids with and without LbL-MPs, also revealed the establishment of well-defined necrotic regions at 7 days (Fig. 5, A), for all conditions (15 000 to 45 000 initial cells, Fig. 5). Moreover, the characteristic cell-proliferative rim at spheroids outer region was readily visualized in both conditions (Fig. 5A and B) Interestingly, despite literature reports describing breast cancer 3D spheroids disruption and loss of stability upon MSCs addition [61], in the lung cancer 3D models produced with cells alone, or with HyA MPs, no changes were observed during the 14 days of culture. Instead, in A549:HF:hBM-MSCs 3D spheroids, a constant density and spherical morphology was observed from day 1 onwards (Figs. 3 and 5). This indicates the importance of hBM-MSCs inclusion in different tumor models since they may trigger differential responses.

The obtained results regarding the establishment of the necrotic core and the characteristic proliferative rim (Fig. 5) indicate that the produced 3D spheroids recapitulate these two major aspects of in vivo solid tumors. These are extremely important hallmarks of solid human
tumors, and their recapitulation shows the potential of the assembled LbL-MPs 3D tri-coculture tumor models. Interestingly mimicking the growth kinetics with cultured cells in a tumor-like microenvironment promotes the establishment of metabolical and phenotypical cell traits [55,62,63]. Adding to this, the accumulation of catabolites and growth factors such as VEGF or HIF-1α, decurrent from increased hypoxic conditions and associated cellular dead can lead to phenotypic alterations in cancer cells and associated MSCs [64]. Such could give rise to pro-tumoral interactions between MSCs and cancer cells as described by Chaturvedi and coworkers, for breast cancer and BM-MSCs co-cultures, in which a hypoxic environment promoted metastasis [65]. The, accumulation of hypoxic and pro-inflammatory factors can also lead to an increased propensity for metastasis and multi drug resistance. These are critical aspects that should be considered when using these 3D microtissues as drug screening platforms [10,55].

To complement live/dead assays and to study whether the insertion of LbL-MPs into cultures elicited a cytotoxic effect, cell viability assays were performed in microtumor models by using Alamar blue (Figure S7, S8 and S9). Viability assays performed in the tested culture conditions showed that the inclusion of bioinstructive LbL-MPs for assembly of microtumors had no effect in cells metabolic activity. Also, the obtained results demonstrated that independently of LbL-MPs amount, no statistically significant variation in cell viability was observable when comparing LbL-MPs spheroids to standard, cell only, 3D-MCTS, both at 7 and 14 days of culture.

Taking into consideration the former results regarding area, circularity, compactness, necrotic core formation and metabolic activity over time, 3D spheroids formed with and initial number of 30 000 cells were selected for subsequent studies. The area and morphology of these spheroids was then characterized in a larger scale as portrayed below, through the analysis of n = 30 spheroids for each condition, to assure a high confidence level for the envisioned drug screening studies.

**Fig. 5.** Fluorescence microscopy micrographs of tri-coculture 3D-MCTS Live/Dead staining. These assays were performed in tri-cocultured A549:HF:hBM-MSCs 3D spheroids and the samples were analyzed by widefield fluorescence microscopy at 7 and 14 days post seeding, revealing necrotic core (red channel) formation in all cell seeding conditions for tri-coculture spheroids, containing (B), or not (A) functionalized LbL-MPs. Green channel: Calcein-AM, Red channel: PI. Yellow channel: merged. Scale bars = 500 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
Fig. 6. Size variation of heterotypic 3D spheroids over time. (A) Area analysis of 3D-MCTS formed with 30,000 cells and assembled with different cell populations. (B and C) Optical contrast micrographs of 3D spheroids morphology obtained at different time points (1, 7 and 14 days post seeding). Scale Bars = 500 μm.
In comparison to monocultured models, dual cocultures and tri-coculture 3D-MCTS with and without LbL-MPs, presented increased contraction (Fig. 6). This observation could be related to ECM components secretion mediated by HF and MSCs, since these two cell types are recognized to contribute immensely to ECM deposition in human TME [39].

In Fig. 7, histological analysis of A549:HF:hBM-MSCs tri-coculture spheroids. (A and B) Spheroids with and without LbL-MPs. (C and D) Internal organization of cells around MPs. Image A, with cells clearly adhering to the surface of LbL-MPs and acquiring an elongated shape (A – red arrows), furthermore a possible necrotic region is observed (red circle). (C and D) At day 7 ECM deposition is already visible in both spheroids occurring however more markedly in tri-coculture 3D spheroids with LbL-MPs (C1 and blue arrow). Scale bar = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

In Fig. 8, CLSM micrographs of tri-coculture spheroids at 7 days of culture. (A) 3D spheroids assembled with and without bioinstructive HyA MPs presented collagen deposition. Green channel: anti-collagen type I-FITC conjugate antibody. Blue channel: DAPI nuclear staining. Scale bars represent 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
3.4. 3D-MCTS histological analysis

H&E and Masson’s Trichrome (MT) staining were performed in A549:HF:MSCs 3D spheroids histological sections to evaluate micro-tumors deep internal organization when cultured with LbL-MPs and to investigate ECM components deposition (e.g., collagen) within the spheroids mass. The histological analysis of 3D microtumors sections, revealed a compact internal cellular organization similar to that of solid human tumors. Compared to other reports with A549 spheroids this result seems to be expected for the established culture times [66]. A closer analysis of spheroids containing LbL-MPs revealed that cells adhering to microparticles were completely spread and extended over their surface, thus establishing contact on one side with the HyA matrix and on the other with surrounding cells (Fig. 7 A, B). Furthermore, regions with LbL-MPs presented a clearly delimited and spherical void space (Fig. 7 A and C).

Regarding tri-coculture spheroids, Masson’s Trichrome (MT) staining that at day 7 showed that collagen deposition in microtumors core containing LbL-MPs is starting to be detected (Blue stained regions Fig. 7C and D). This result is in accordance with the increased contraction rates observed in tri-coculture spheroids when compared to its A549 monoculture counterparts. To further complement collagen deposition, immunofluorescence labeling of collagen I in whole 3D spheroids mass was performed.

3.5. Immunocytochemistry analysis of 3D-MCTS ECM components

Collagen I immunocytochemistry analysis was also performed in A549-HF-MCs 3D spheroids to further analyze collagen deposition and if the increased cohesion observed by optical microscopy analysis of tri-coculture models could be associated with the deposition of tumor-ECM components. Collagen is an abundant structural component of the human ECM, is constituted by several diverse types of which collagen type I is the predominant form in most tissues [67]. During tumor progression established interactions between fibroblasts and cancer cells can lead for example to increased matrix deposition and collagen cross-linking, resulting in changes in ECM architecture [67]. This is characterized in part by increased collagen re-deposition/crosslinking,
and ultimately leads to matrix stiffening. From a therapeutic perspective, this event strongly contributes for the establishment of a barrier to chemotherapy penetration and hinders their efficacy in deep tumor regions [68]. While the role of collagen in the TME has not yet been fully elucidated [67], increased collagen deposition in 3D spheroids has been linked with increased interstitial pressure. Moreover, an increased collagen deposition has also been connected with an increased metastatic potential in breast cancer [69].

The results obtained by immunocytochemistry revealed that at day 7, collagen deposition was clearly visible in Lbl-MPs 3D tri-coculture microtumors mass (Fig. 8). Such leads to the formation of a network of collagen fibrils over the 3D spheroid volume and can contribute to spheroids cohesion and the establishment of a necrotic core. Moreover, collagen deposition and fibril alignment are recognized as key factors involved in cancer cell invasion of tissues resulting of microenvironment remodeling by cancer cells, being associated with the development of pro-metastatic cell phenotypes in breast, lung and prostate cancer [69–71].

Overall, these findings demonstrate 3D spheroids ability to closely mimic key solid tumor features through de novo collagen deposition. It is important to emphasize that the produced collagenous matrix can act both as a structural component and as a store house of cellular signaling factors (e.g., growth factors) [72] during in vitro culture. Following this de novo tumor-ECM characterization, we aimed to investigate the role of Lbl-MPs inclusion in the overexpression of key cancer cells surface markers, in particular, the cluster of differentiation 44 (CD44), which is widely recognized by its ability to interact with ECM hyaluronan.

3.6. Flow cytometry analysis of CD44

Several studies reported that in contact with hyaluronic acid-rich substrates, cancer cells increase their expression of the CD44 cell surface receptor [72,73]. Increased CD44 expression has been intimately connected with cancer multi-drug resistance (MDR) and with the development of cancer stem cell-like phenotypes [74,75]. As such, the effect bioinstructive Lbl-MPs on CD44 cell surface receptor expression in mono-, dual and tri-coculture 3D microtumors was evaluated (Fig. 9).

The obtained results show a clear increase in CD44 expression in mono-, dual and tri-coculture 3D spheroids upon the inclusion of bioinstructive Lbl-MPs emphasizing their importance as tumor mimicking in vitro platform. In particular, dual cocultures of A549: HF and tri-cocultures experienced approximately a ~2 fold increase in the percentage of CD44+ cells when adhered to Lbl-MPs in vitro. Interestingly, dual cocultures of A549:hBM-MSCs inherently express high levels of CD44+ cells. Taking into consideration the A549:hBM-MSCs 1:10 ratio, this high expression of CD44 can be related both with the inclusion of CD44+ hBM-MSCs [31], and can also be attributed to the interactions established between cancer-MSC populations, which lead to this increase as widely reported in the literature [73,76,77]. Overall, from a cancer biology perspective an increase in CD44 during tumor development is an observed event that is herein recapitulated with the inclusion of Lbl-MPs. These findings corroborate the bioinstructive role of Lbl-MPs in 3D-MCTS, evidencing their ability to act as an ECM mimetic support, in this case via HyA. It is important to emphasize that due to Lbl-MPs morphology (Fig. 10), extending themselves over the exposed surfaces as observed in histological analysis (Fig. 7).

Furthermore, over the course of 14 days, the fluorescence signal emitted by DiD (the cell marker associated hBM-MSCs), was clearly visible, denoting the presence of MSCs. The obtained high-resolution micrographs also demonstrate an internal organization of mesenchymal stem cells into hotspots inside tri-coculture spheroids. These clusters are surrounded by numerous A549 cancer cells and dermal fibroblasts with no apparent well-defined spatial organization (Fig. 11, at 14 days). Moreover, as shown in Fig. 11A and B, in both cell-cell aggregated or Lbl-MPs structured tri-coculture 3D models, MSCs demonstrated a tendency to condense into MSCs rich agglomerates along time. Interestingly, at early time points (3 and 7 days), hBM-MSCs are still randomly dispersed in Lbl-MPs 3D microtumors (Fig. 11, B4 and B8). Yet, at 14 days of culture, a clear stem cells agglomerate was observed in 3D spheroids center (Fig. 11, B12).

These interesting findings led us to evaluate MSCs migration into 3D tumor models also after their formation, as this would better recapitulate the in vivo scenario of stem cells tropism to tumor sites [34,81]. Cell tracking analysis clearly showed that upon addition of Dd-stained hBM-MSCs to pre-formed 3D spheroids, these cells were able to migrate to the interior of mono- and coculture microtumor masses in all tested conditions, after only 24 h of incubation (Fig. 12). Furthermore, it is interesting to denote that despite hBM-MSCs presence in dual-coculture and tri-coculture models, the administered hBM-MSCs still adhered, penetrated and migrated in these spheroids. In all the conditions tested, hBM-MSCS organized into clusters in the microtumors mass (Fig. 12A and B, C,D at 168 h), providing important evidences for further fundamental studies or therapeutic approaches [78], including such as those based on the use of MSCs as Trojan horses for chemotherapeutics/nanoparticles administration to tumors [82]. To the best of our knowledge this is the first time that a tri-coculture and cell tracking analysis with hBM-MSCs has been performed in vitro. Such findings also emphasize that the bioengineered 3D tumor models with Lbl-MPs recapitulate the features of in vivo tumors by providing a proper niche for MSCs to settle, rearrange spatially and interact with other cells of the tumor microenvironment.

Having established the tumor-like features of A549:HF:hBM-MSCs 3D spheroids cultured in Lbl-MP platforms and the successful inclusion of hBM-MSCs in the microtumor mass, the screening of a model
chemotherapeutic compound (Doxorubicin) cytotoxic activity in these models was then investigated with the aim to draw relevant conclusions regarding the influence of hBM-MSCs and LbL-MPs inclusion in 3D in vitro lung tumor models.

3.8. Chemotherapeutics drug screening in bioinstructed 3D-MCTS

Interactions between diverse populations present in the TME are known to be of paramount importance for the performance of chemotherapeutics. In fact, the close interactions established between stromal and cancer cells through direct contact or indirect soluble cues such as cytokines, chemokines and growth factors ultimately lead to altered metabolic profiles, signaling pathways, and invasive behavior [83]. Moreover, this communication incites drug resistance through several cancer cells defense mechanisms that include a decreased uptake of water soluble chemotherapeutic compounds, and the modulation of cancer cells metabolic regulation either through ‘self-genetic’ mutation or interaction with cellular components of the tumor microenvironment (TME) (e.g., fibroblasts, immune system cells and MSCs). Such unique crosstalk between cancer cells and their TME may lead to a decreased cell cycle arrest, increased repair of DNA damage, reduced apoptosis, and an increase in energy-dependent efflux of hydrophobic chemotherapeutics such as Doxorubicin is also often obtained [84,85]. The result is an area were therapeutic agents’ penetration is possibly hindered. From a bioengineering point of view, modeling these hallmarks of in vivo tumors within 3D in vitro tumor models is highly desirable.

The coculture of cancer cells with HF and hBM-MSCs and the existence of a necrotic core signalizes the lack of nutrient and medium penetration into 3D microtumors deepest regions. By establishing a 3D coculture model capable of representing in vitro A549 cancer cells in vivo like interactions with HF and hBM-MSCs, we aimed to recapitulate tumor cellular heterogeneity [40,42,78,86]. Furthermore, the inclusion of hyaluronan aimed to recapitulate tumor-ECM components. In fact, in lung cancer TME, HyA is associated with poor tumor cell differentiation and higher recurrence rate when present in elevated quantities [17]. Moreover, interactions of LbL-MPs with stromal cells such as HF or MSCs, have also been connected as possible roots towards establishment drug resistance phenotypes [20].

Drug screening assays were then performed to access the influence of HyA and heterotypic tri-cocultures in comparison to more simplistic 3D in vitro tumor models formed only by cancer cells or by non-functionalized MPs. For this purpose, Doxorubicin (Dox) was administered to 3D spheroids cultivated over a period of 7 days, to allow the establishment of cell-HyA and cell-cell contacts. The inclusion of LbL-MPs into 3D spheroids generally originated a slightly higher cell viability when compared to control 3D microtumors or NT-MPs spheroids (Fig. 13A–C). The obtained higher viability in tri-coculture with HyA MPs was more pronounced in A549 monotypic spheroids (*p < 0.05). The lack of a significant increase in resistance in dual and tri-coculture LbL-MPs spheroids could be correlated with the inclusion of stromal cells and added models’ complexity. Importantly, non-functionalized PCL MPs (NT-MPs) exhibited a higher susceptibility to Dox in comparison to tri-coculture 3D spheroids (Fig. 13A–D, 3D-MCTS with NT-MPs).
MPs), which could be associated to a lack of cellular adhesion and bioactivation, since these microparticles only present a plasma treated PCL surface and not a bioinstructive layer. These findings emphasize the successful inclusion of HyA component and its biological effects.

The acquisition of higher resistance to Dox in LbL-MPs 3D spheroids could be related to several factors, including the formation of more compact spheroids via increased matrix deposition or via increased expression of CD44 markers as demonstrated by FCM analysis. Previous works by Han and coworkers, 2016, demonstrated that A549 spheroids cells assembly was mediated through a CD44-dependent mechanism [79]. The possible establishment of cell-cell interactions between HF and A549 cells could be directly correlated with the lower Dox cytotoxicity index in these cultures, closely mimicking events in in vivo tumors [87].

In an overall analysis, cell-cell agglomerate 3D spheroids and LbL-MPs 3D models cultured in dual and tri-coculture, generally presented higher cell viability at the highest tested dose (17 μM Dox), than their monoculture counterparts. In addition, an all-round characterization of 3D spheroids assembled with half mass of LbL MPs (0.0125 mg) was performed (Figure S11 to S14). In summary, the obtained results demonstrate that the use of a lower mass approximated 3D LbL MPs spheroids chemotherapeutic response to that of standard 3D-MCTS, indicating that the optimized amount of LbL MPs (0.025 mg) may be a more suitable model to recapitulate the features of resistant tumors.

It is important to emphasize that these results are obtained with bone marrow derived MSCs and that MSCs of different origins may originate differential responses to chemotherapeutics as was recently reviewed [34]. Paradoxically, such interactions were found to be correlated with the establishment of pro-tumoral or anti-tumoral interactions with cancer cells. Hence, we aimed to further test this hypothesis, by establishing a non-physiological A549:hBM-MSCs ratio of 1:1, having this model been subjected to the same culture conditions and Dox concentrations. The obtained results evidence a pro-tumoral role of hBM-MSCs in A549 lung cancer cells when present in low ratios (A549:hBM-MSCs 10:1 ratio), (Fig. 14A). On the contrary, when A549 and hBM-MSCs were cultured in a 1:1 ratio a higher susceptibility to Dox was observed (Fig. 14B). Such findings demonstrate, the variable and important role of MSCs in the tumor microenvironment [30,41],

Fig. 11. Widefield fluorescence micrographs of tri-coculture 3D spheroids stained with cell tracking dyes demonstrated a tendency in hBM-MSCs to colocalize into the interior of the spheroid. (A) Cultured hBM-MSCs formed visible cell clusters at the inner regions of the spheroids over time, taking 7 days in 3D-MCTS not containing LbL-MPs, (B) and 14 days in microtumors formed by cell agglomerates and bioinstructive LbL-MPs. Scale bar = 500 μm.
emphasizing the importance of including this cell type in the development of in vivo-like preclinical drug screening platforms to achieve a more robust recapitulation of the in vivo tumor microenvironment.

In summary, the different data obtained through the newly established co-culture model of A549, with HF and hBM-MSCs, corroborates the necessity of further analyzing the cooperation of TME stromal cells with cancer cells in in vitro 3D models. It is important to mention that this was also the first time a 3D model comprising these tri-cocultures and HyA matrix in bioinstructive microparticles was developed and used as a testing platform for evaluation of chemotherapeutics anti-cancer performance, in TME relevant conditions. In the future, additional screening performed with other clinically used chemotherapeutics that have different mechanisms of cytotoxicity could further provide important insights into the employment of LbL-MPs and hBM-MSC models as in vitro tumor surrogates.

4. Conclusions

By taking advantage of a combination of coculture conditions, based on previous literature reports, an easy to assemble composite 3D tri-coculture model containing key TME populations such as HF and hBM-MSCs was established by using spherical microparticles as bioinstructive anchoring substrates. The inclusion of HF and MSCs stromal cells facilitated the formation of a characteristic necrotic core, the appearance of which is known to promote resistance to therapy in vivo. Moreover, the inclusion of bioinstructive microparticles allowed the addition of HyA, a main component of tumor-ECM that is recognized to contribute to cells response to chemotherapeutics.

Overall the inclusion of a small percentage of bone marrow derived MSCs appears to result in a positive outcome in overall microtumors cell viability, improving the ability of A549 cancer cells to form compact spheroids and resist better to Doxorubicin-induced cytotoxicity, in specific cell-cell ratios. In the foreseeable future these models may also be used for further evaluating MSCs migration, clustering, or therapeutic effectiveness as trojan horses.

In conclusion, the in vitro generated bioinstructed 3D-MCTS exhibit characteristics associated with in vivo tumors such as development of highly dense cell masses and de novo matrix deposition. Such hybrid 3D spheroids containing LbL-MPs served as an effective tool for tackling one of the main flaws of spheroids-based models, i.e, the integration of pre-existing ECM derived components. The assembled tri-coculture in vitro models can be used to screen novel therapeutic compounds for...
lung cancer in more in vivo-like conditions. Due to its versatility, this enabling technology can also be used to study different combinations of cell populations and more complex ECM domains can be easily included, thus allowing to evaluate their specific functions and effects in cancer cells survival with different treatments.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biomaterials.2018.09.007.

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