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Multifunctional laminarin microparticles for cell adhesion and expansion

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Abstract

Microfabrication technologies have been widely explored to produce microgels that can be assembled in functional constructs for tissue engineering and regenerative medicine applications. Here, we propose microfluidics coupled to a source of UV light to produce multifunctional methacrylated laminarin microparticles with narrow distribution of sizes using photopolymerization.

The multifunctional microparticles were loaded with platelet lysates and further conjugated with an adhesive peptide. The adhesive peptides dictated cell adhesiveness to the laminarin microparticles, the incorporation of platelet lysates have resulted in improved cell expansion compared to clear microparticles.

Overall, our findings demonstrate that multifunctional methacrylated laminarin microparticles provide an effective support for cell attachment and cell expansion. Moreover, expanded cells provide the link for microparticles aggregation resulting in robust 3D structures. This suggest the potential for using the methacrylated laminarin microplatforms capable to be assembled by the action of cells to rapidly produce large tissue engineered constructs.

Keywords

Microfluidic; microcarrier; microgels; platelet lysates; methacrylated laminarin; injectable scaffolds

1 Introduction

Living tissues are hierarchically organized three-dimensional (3D) structures composed of multiple cell types and extracellular matrix (ECM) that provides structural and biochemical support (Discher, Mooney, & Zandstra, 2009). Thus, effective strategies to engineer living constructs that mimic native tissues require the development of structures with well-defined spatial distributions of different cells embedded in ECM-like materials.

Current strategies for tissue and organ development include “bottom-up” tissue engineering (TE), that consists in the assembly of smaller units to build a 3D construct and “top-down”

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approaches, involving a scaffold-based cell seeding. If classical TE approaches have followed a “top-down” strategy, the “bottom-up” organization of cell-laden small hydrogel units that mimic the living tissue architecture, have gained increased attention in recent years (Y. B. Hu et al., 2017) (Kim et al., 2014) (Cavalieri, Postma, Lee, & Caruso, 2009) (Custodio, Cerqueira, Marques, Reis, & Mano, 2015) (Neto, Levkin, & Mano, 2018).

To engineer the miniaturized structures for cell culture, so-called building blocks, different microfabrication strategies have been explored (Custodio et al., 2015) (Mao et al., 2017) (Neto et al., 2016). Among them, microfluidics is a promising technology that enables a precise control and manipulation of fluids at microliter/nanoliter range, being the most promising approach to produce monodisperse functionalized microgel capsules and particles (Q. Wang, Liu, Wang, Zhu, & Yang, 2015) (Y. D. Hu, Azadi, & Ardekani, 2015) (Guerzoni et al., 2017) (Ma, Neubauer, Thiele, Fery, & Huck, 2014).

Laminarin is a natural polymer obtained from brown algae with low molecular weight and low viscosity (D. Wang et al., 2017). These properties make this polymer particularly appealing to be processed using microfabrication techniques. Photopolymerizable hydrogels from methacrylated laminarin (MeLam) have recently been proposed as an enabling platform to encapsulate human stem cells that remain fully viable for several days. (Custodio, Reis, & Mano, 2016).

In this work, we report a simple and efficient microfluidic approach to produce monodisperse MeLam microparticles with encapsulated platelet lysates (PL). PL loaded scaffolds and microparticles have been successfully used to improve the biological performance of biomaterials. PL supplementation of cell culture media is also gaining an increasing interest as animal serum substitutes, especially for cells that need to be implanted in the patient (Custodio et al., 2014) (Oliveira et al., 2016). Herein, the encapsulation of PL was used to improve cell attachment and promote cell expansion in the MeLam microcarriers.

The methacrylate groups present on the photopolymerizable laminarin backbone could act also as anchoring sites for the immobilization of thiolated molecules. In the present work, the microparticles were functionalized with thiol-biotin molecules for the subsequent binding of biotinylated RGD molecules in an attempt to accelerate and enhance cell adhesion.

Using a biocompatible and biodegradable microplatform as support for cell culture and expansion, we provide simultaneously an injectable system that can be used to fill tissue defects in TE strategies using minimally invasive strategies. We show that the multifunctional microparticles present great promise in supporting the long-term culture of cells and can then be used as building blocks to create a 3D tissue constructs.

2 Materials and Methods

2.1 Synthesis and characterization of methacrylated laminarin

Methacrylated laminarin (MeLam) was modified by a common chemical reaction following the protocol previously described (Custodio et al., 2016). Briefly, MeLam was synthesized by reacting laminarin (Molecular weight $\approx$ 6kDa) (Carbosynth, U.K.) with glycidyl methacrylate (Acros Organics, Germany). Laminarin (1g) and 4-(*N,N*-dimethylamino)pyridine (DMAP) (167 mg) (Acros Organic, Germany) were dissolved in 10 mL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Germany) under nitrogen atmosphere. By varying the amount of glycidyl methacrylate it was possible to manipulate the degree of modification; low degree of modification was obtained by adding 2.9×10^{-3} mol of glycidyl methacrylate and high degree of modification by adding 5.1×10^{-3} mol of glycidyl methacrylate. The mixture was stirred at room temperature for 48 hours protected from light, being stopped by adding HCl solution (37%) (Sigma-Aldrich, USA) to neutralize DMAP. Subsequently, the solution was purified by dialysis using a benzoylated membrane (2000 MWCO) (Sigma-Aldrich, USA) for at least 7 days against distilled water. The final product was freeze-dried and stored at room temperature until further use.

Degree of substitution (DS, fraction of modified hydroxyl groups per repeating unit) was inferred from ^1H NMR spectroscopy (Bruker Avance III (300 MHz)) by comparing the intensities of the peak correspondent to the methyl group signal of the acetyl group ($\sim$ 2 ppm (IAc)) and the polymer backbone region $\sim$ 3–5.5 ppm (ILam). The following formula (Eq. 1) was used to calculate the DS:

$$DS = \frac{I_{AC}}{I_{Lam}} \quad \text{Equation 1}$$

2.2 Fabrication of MeLam microparticles

Water-in-oil droplets were formed within the microchannels of a hydrophobic droplet junction chip with header (190 μm etch depth) (Dolomite, UK) (Fig. 1B) by means of water-soluble MeLam as a dispersed phase. For the dispersed phase, MeLam was dissolved in phosphate buffered saline (PBS, pH 7.4) (Sigma, USA) at a concentration of 15% (w/v) with 0.5% (w/v) 2-hydroxy-4-(2-hydroxyethoxy)-2-methylpropiophenone (Sigma, USA) and biotin-PEG-thiol (Polypure AS, Norway) (0.5 mg/mL). The previously prepared solution was loaded into a plastic syringe and connected to the inlets of the microfluidic chip using fluorinated ethylene propylene (FEP) tubing. For the continuous phase, mineral oil (Fisher, USA) was loaded into the same type of syringe. Syringe pumps (Harvard Apparatus, USA) were used to inject fluids at controlled flow rates into the microfluidic chip (Fig. 1A), the flow rates were set to be 8 $\mu\text{L}/\text{min}$ for the dispersed phase and 160 $\mu\text{L}/\text{min}$ for the continuous phase. Upon formation in the flow-focusing device, droplets were photopolymerized upon irradiation with a UV light source (Omnicure S2000, Canada) to form microparticles. The outlet tubing (0.5 mm of diameter) was coiled to make a spiral microchannel ensuring that the microdroplets are kept for at least 60 seconds under UV light (6.12 W/cm^2) for the

efficient crosslinking of microparticles (Fig. 1C). The MeLam microparticles were collected in a falcon tube (15 mL) and the mineral oil removed after centrifugation.

To produce MeLam microparticles with encapsulated platelet lysates (PL) the protocol was slightly changed by including PL in the dispersed phase. Briefly, PL (25% v/v) was mixed with the MeLam solution previously dissolved in PBS and loaded into the plastic syringes.

2.3 RGD functionalization of MeLam microparticles

To assess the functionalization with thiol-PEG-biotin, the modified MeLam microparticles were incubated with DyLight 488 Streptavidin (BioLegend, USA) prepared at a concentration of 10 µg/ml in PBS for 15 minutes. Subsequently the microparticles were washed with PBS and observed under fluorescence microscopy (Axio Imager M2, Zeiss, Germany). Polymeric microparticles with covalently attached biotin are herein proposed as versatile targeting vehicles for multiple biomolecules; in this work the microparticles were functionalized with the tripeptide Arg-Gly-Asp (RGD) to increase cell adhesion. Briefly, biotinylated microparticles were incubated with purified streptavidin (Promega, USA) (25 µg/ml) in PBS under gently agitation for 15 min at room temperature. A washing step was then performed to remove unbound streptavidin. Finally, the MeLam microparticles were incubated with biotinylated RGD (25 µg/mL) (AnaSpec, USA) in PBS under gently agitation for 15 min at room temperature. The MeLam microparticles were finally washed with PBS to remove unbound biotinylated RGD.

2.4 Scanning electron microscopy (SEM)

SEM was performed as a means to evaluate the morphology and porosity of the MeLam microparticles produced by microfluidics. The microparticles were dried at 25°C for 3 days, sputtered with gold and evaluated by SEM (Hitachi SU-70, Japan).

2.5 Studies on the release of PL

Aliquots of the multifunctional MeLam microparticles were suspended in 5 mL PBS, samples were gently shaken at 60 rpm in a water bath at 37°C. At defined time intervals, 500 µL of PBS were removed and replaced with 500 µL of fresh PBS. The removed supernatants were stored frozen until required and were then assayed for total protein content using the Micro BCA assay kit. Briefly, 50 µL of the collected samples were diluted in 100 µL of PBS, mixed with 150 µL of the Micro BCA working solution and incubated for 2 hours at 37°C. Afterwards, the quantity of protein was measured by the absorbance at 592 nm in a microplate reader (Synergy HTX multi-mode reader, Biotek Instruments, Inc, USA). The protein release profile was calculated following equation (2) (Che et al., 2015):

$$\text{Cumulative PL release(\%)} = \frac{V_e \sum_{i=1}^{n-1} C_i + V_0 C_n}{m_{PL}} \times 100 \quad \text{Equation 2}$$

Where is $V_e = 500 \mu\text{L}$; $V_0 = 5 \text{ mL}$; C_i is the concentration of total protein released from MeLam microparticles at the time and m_{PL} is the weight of PL used for the release.

ELISA assay (ThermoFisherScientific, USA) was also performed to evaluate the release of transforming growth factor (TGF- β 1) and vascular epithelial growth factor (VEGF) from the MeLam microparticles. The assay was performed according to the manufacturer's standard protocols. The optical density values were measured using a Synergy HTX multi-mode reader (Biotek Instruments, Inc, USA) set at 450 nm.

2.6 Cell culture on MeLam microparticles

Mouse fibroblasts cells (L929, ECACC) were used to verify the potential of MeLam microparticles for cell adhesion and expansion. L929 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Thermo Scientific, USA) supplemented with 10% of fetal bovine serum (FBS, Thermo Fisher Scientific, USA) and 1% antibiotic/antimycotic (Thermo Fisher Scientific, USA) at 37°C under 5% CO₂. L929 cells were seeded at a density of 50 cells per microparticle. 10 μ l of the particle suspension were pipetted to a glass slide and the microparticles counted under a light transmitted microscope (Zeiss, Germany). The seeded microparticles were kept in culture under gently agitation (150 rpm) at cell culture conditions (5% CO₂, 37°C) for 11 days. At pre-determined time points, cell morphology, viability and proliferation were assessed. The assembly process of cultured microparticles was followed and imaged at day 11.

2.7 Cell morphology analysis

Phalloidin and DAPI staining were used to visualize actin cytoskeleton and to label the nuclei, respectively. The assay was conducted as outlined by the supplier's protocol (Sigma, Germany). Briefly, cultured MeLam microparticles were washed with PBS, fixed in 4% formaldehyde/PBS (v/v) for 1h at RT and washed extensively in PBS to remove all traces of the fixative. Cells were then incubated with 50 μ g/mL of fluorescent phalloidin-conjugate solution in PBS for 45 min at RT. DAPI labeling solution 0.5 μ g/ml was incubated for 5 min at RT. The MeLam microparticles were then washed in PBS to remove remaining staining solutions and imaged using a fluorescent microscope (Axio Imager M2, Zeiss, Germany).

2.8 DNA quantification

Cell proliferation on the multifunctional MeLam microparticles was determined by DNA quantification using a fluorimetric double-stranded DNA (dsDNA) quantification kit (PicoGreen, Invitrogen, USA). This assay allows the measurement of the fluorescence produced when PicoGreen dye is excited by UV light while bounded to dsDNA. After each time-point, cells were lysed by osmotic and thermal shock and the supernatant used for dsDNA content analysis. Briefly, samples collected after each time point were washed with PBS and immersed in 1 mL of ultrapure water, frozen for at -80 °C, thawed at room temperature, and sonicated for 30 min. Fluorescence was measured on a microplate reader (Synergy HTX multi-mode reader, Biotek Instruments, Inc, USA). The dsDNA amount was calculated from a standard curve.

2.9 MTS Viability Assay

The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2Htetrazolium) assay (Promega, USA) was performed to evaluate cell

metabolic activity. Briefly, after each time period, culture media was removed and cells were washed with PBS solution. The MTS solution 1:10 ratio in PBS was added to the cell cultured particles and incubated for 4 h (37°C, 5% CO₂). After the incubation period, the optical density (OD) was read at 490 nm in a microplate reader (Synergy HTX multi-mode reader, Biotek Instruments, Inc, USA).

2.10 Statistical analysis

All results were subjected a statistical analysis and results were presented as mean $\pm$ standard deviation. Statistical analysis of results was performed using Student's t-test, with a significant level of 95% ($p < 0.05$).

3 Results and Discussion

3.1 Synthesis and Characterization of Methacrylated Laminarin

Laminarin is a low-molecular-weight polysaccharide and bioactive compound present in brown algae (D. Wang et al., 2017). The abundance of hydroxyl groups in the laminarin structure may be used to insert a polymerizable moiety or to chemically bind a bioactive agent. The modification of laminarin using glycidyl methacrylate has recently been proposed and was explored in this work (Custodio et al., 2016). Briefly, methacrylated laminarin was synthesized as a hydrogel precursor by taking advantage of the functionality of the hydroxyl groups in laminarin as well as the reactivity of the epoxy group in glycidyl methacrylate (Fig. 2A). ¹H NMR spectroscopy confirmed the modification of laminarin through the appearance of three new peaks, the peaks at $\delta = 5.7$ ppm and $\delta = 6.1$ ppm correspond of vinylic protons (C=CH₂) and the peak at $\delta = 1.9$ ppm corresponds of the protons of methyl group (CH₃) (Fig. 2B, 2C and 2D). Integration and normalization of the methyl group peak in the methacrylate segment in relation to the hydrogen peaks of the laminarin backbone (3 ~ 5.5 ppm) provides consistent means for calculating the degree of substitution of hydroxyl groups in laminarin by the methacrylate group. The average degree substitution increased from $\approx 30\%$ (Low-MeLam) to 60% (High-MeLam), by adding 7% (2.9×10^{-3} mol) and 14% (5.1×10^{-3} mol) (v/v) glycidyl methacrylate to laminarin in the modification procedure.

3.2 Fabrication of Methacrylated Laminarin microparticles

Based on the promising results of MeLam hydrogels as cell culture platforms and on the intrinsic low viscosity of this polymer we hypothesized that microfabricated MeLam hydrogels could provide effective microplatforms for cell culture and expansion. Microfluidics has proved to be efficient in producing highly monodisperse microparticles with spherical shape (Guerzoni et al., 2017) (Ma et al., 2014) (Cha et al., 2014) (Zhao et al., 2016). In this work laminarin was processed in a microfluidic flow-focusing device, monodispersed droplets of MeLam were formed by the shear forces exerted from the oil phase using a hydrophobic droplet junction chip, the droplets were finally crosslinked upon irradiation with a UV light source. (Fig. 1). Using microfluidics, droplet formation is easily tuned by adjusting the channel geometry and flow rates of the continuous and dispersed phases, offering great control over the size, shape and morphology of the microparticles. The ideal size for smooth cell microcarriers varies between 100 and 300 μm (H. Hauser, 2014). The smaller is best suited for stirring flasks, whereas the higher sedimentation rates of the

larger make them suitable for more static systems. In the present work, different flow rates were tested being noticeable that with an increase in the ratio disperse phase/continuous phase (Q_D/Q_C) corresponds to a decrease in droplet diameter. The precise control of flow rates (160 $\mu\text{L}/\text{min}$ for continuous phase, 8 $\mu\text{L}/\text{min}$ for disperse phase) guaranteed an average size of 100 μm for the MeLam microparticles with an average size of 300 μm after swelling (Fig. 3). The spherical shape, monodispersity and smooth surface of microparticles was observed by SEM analysis for both degrees of modification microparticles (Fig. 3G and 3H).

3.3 Cumulative release of platelet lysates from MeLam microparticles

Hydrogel microparticles have demonstrated great potential as drug delivery systems due to facile incorporation and finely tuned release of biomolecules (Guerzoni et al., 2017) (Lima, Sher, & Mano, 2012). The MeLam cell microcarriers here developed act as a support for cell attachment and have also the capability to deliver bioactive factors to further control cell function, such as proliferation and differentiation. In this work, platelet lysates (PL) were used as a source of growth factors (GFs) to improve cell adhesion and promote cell expansion. To achieve that, PL (25% v/v) was incorporated in the MeLam solution and this mixture was processed as previously described, using the microfluidic apparatus. The release profile of encapsulated PL in high and low MeLam microparticles was followed up to 14 days, and the cumulative protein release is shown in Fig. 4A. High MeLam microparticles exhibit a release of $40.31 \pm 0.27\%$ of the total encapsulated protein after 12 hours while the low MeLam exhibit a release of $22.54 \pm 1.13\%$ after the same period. At the end of 14 days, $56.89 \pm 1.03\%$ of the total protein was released from the high MeLam microparticles, while only $35.31 \pm 2.73\%$ of the total encapsulated protein was released from the low MeLam microparticles. Protein release rate depends on the solubility, diffusion and biodegradation of the encapsulation matrix. The sustained duration of release of proteins from both type of microparticles renders this an ideal delivery vehicle for PL. The small difference in the release profile for low MeLam and high MeLam may be related to the methacrylation degree. Due to different levels of substitution, the chemical interactions between the polymeric network and proteins will be different, which may explain the different release profiles. This can be explained by covalent or electrostatic interactions between the matrix of the microparticles and proteins. Ngyyen and co-workers studied the influence of different degrees of methacrylation and the ability to bind and release proteins and GF (Nguyen, McKinney, Miller, Bongiorno, & McDevitt, 2015). They demonstrated that decreasing the degree of methacrylation increases protein binding. This is in accordance with our results, where the low MeLam microparticles provide a slightly more sustained release of proteins compared to the high MeLam. The amount of PL released from low MeLam microparticles and high MeLam microparticles was 20% and 40% respectively after 10h (Figure 4A). Maximum release observed over the experiment (14 days) was ~30% low MeLam microparticles and 55% for high MeLam microparticles. These results suggest that protein delivery systems based on MeLam microparticles, can be easily modulated by adjusting the degree of modification of laminarin. PL are rich in several chemokines and GFs such as platelet derived growth factor isoforms (PDGF-AA, -AB and -BB), transforming growth factor- β (TGF- β), insulin-like growth factor-1 (IGF-1), vascular endothelial growth factor (VEGF) and bone morphogenetic protein 2, -4 and -6 (BMP-2, -4, -6). Several works have suggested that PL are a valuable, non-xenogenic alternative to

animal derived serum in cell culture (Bieback, 2013) (Turner, Thiele, & Stegemann, 2017) (Burnouf, Strunk, Koh, & Schallmoser, 2016) or combined with biomaterials (Santos, Sigurjonsson, Custodio, & Mano, 2018) (Oliveira, Santo, Gomes, Reis, & Mano, 2015). Physiologically, platelets are known to deliver a broad spectrum of GFs and have a main role in wound healing. In the present work, the encapsulation of PL and subsequent sustained release from the MeLam microparticles, was proposed as a platform to enhance cell adhesion and growth. Platforms for the controlled delivery of GFs should be designed to deliver multiple biomolecules at different rates to promote synergistic effects on the adhesion, proliferation, migration and differentiation of cells. In the present work a complex mixture of GFs was immobilized in the MeLam matrix through electrostatic interactions between oppositely charged groups on GFs and polymer chains on the substrate. VEGF and TGF- β 1 have a key role in cell proliferation and angiogenesis, processes that guarantee long-term survival and function of 3D tissue constructs. It was suggested that the dual sustained release of VEGF and TGF- β s enhances the formation and maturation of newly formed blood vessels compared with that of single VEGF release (Ferrari, Cook, Terushkin, Pintucci, & Mignatti, 2009). These factors are attached to the ECM via specific interactions and act in spatio-temporal gradients to regulate vessel density, size and distribution (Shih et al., 2003). The synergistic effect of both GFs in osteogenesis has also been reported, TGF- β 1 stimulates matrix formation including collagen and osteonectin while VEGF plays an important role in bone metabolism and affects both osteoblasts and osteoclasts (Hayrapetyan, Jansen, & van den Beucken, 2015) (Kuroda, Sumner, & Virdi, 2012). In this study, the release profile of VEGF and TGF- β 1 was assessed by ELISA assay (Fig. 4C and 4D). VEGF and TGF- β 1 release from MeLam microparticles followed a similar pattern to the total protein release profile with a slight low decrease in mass released corresponding to a lower methacrylated polymer. A burst release was observed for VEGF (~60%) in both high and low MeLam microparticles, followed by a much lower rate of factor release (Fig. 4C). TGF- β 1 shows a smaller burst release of ~40%, with 80% of release after 1 hour (Fig. 4D). Both high MeLam and low MeLam have a sustained and prolonged release over 14 days. Collectively, the results show sequential delivery of the GFs; with initially, high levels of VEGF being released followed by TGF- β 1. A main objective herein proposed is to show for the first time the potential of multifunctional MeLam microparticles as a platform for cell expansion. Considering enhanced release of PL proteins and GFs from the high MeLam particles at the first hours of incubation, those microparticles were selected for the subsequent studies as cell culture platforms.

3.4 RGD functionalization of MeLam microparticles

Due to the high stability and specificity between biotin and streptavidin (SaV), this complex has been a very powerful tool in the study of biological systems being used for chemical conjugation of biomolecules (e.g. antibodies, peptide sequences and GFs) (Chivers, Koner, Lowe, & Howarth, 2011). Taking advantage of the SaV-biotin pair, the focus in this work was the immobilization of biotinylated RGD to enhance cell adhesion to the MeLam microparticles (Fig. 5A). The MeLam microparticles were first conjugated with biotin-PEG-SH by reaction with the alkene groups presents in microparticles *via* Michael type reaction. A second modification step was performed to create a coating of SaV in the MeLam microparticles, followed by an incubation with biotin-RGD. A major advantage of this

system, is the possibility to conjugate the microparticles with an array of different biotinylated molecules. The effective modification of the MeLam microparticles with biotin-PEG-SH was evaluated by fluorescence microscopy using fluorescent-labeled SaV and the unmodified microparticles as a negative control. The fluorescence images demonstrate the efficient conjugation the MeLam microparticles with SaV, hence confirming the effective previous modification with biotin. (Fig. 5B and 5C). The last step of the functionalization was the addition of the RGD-biotin. RGD is a peptide sequence (Arg-Gly-Asp) that constitute a major recognition system for cell adhesion (Fig. 5D and 5E) (Ruoslahti, 1996).

3.5 L929 fibroblast adhesion and expansion on MeLam microparticles

Microcarrier beads of different materials, have been widely used to culture anchorage-dependent cells. Microcarriers have innumerable advantageous when compared with the conventional cell culture systems. The low cost and great surface-to-volume ratio allow the culture of high cell numbers, eliminating multiple trypsinization steps (Sun et al., 2015). Recently, Soure and co-workers studied the effect of PL on the expansion of mesenchymal stem cells (MSCs) derived from umbilical cord using plastic microcarriers under dynamic conditions. (de Soure et al., 2017) Their results demonstrated the advantages of the use of PL for the effective expansion of MSCs in a xenogeneic-free microcarrier-based system. In the present study, it is proposed the fabrication of MeLam microparticles with encapsulated PL to be used as microplatforms for cell adhesion and expansion. L929 mouse fibroblasts were used to evaluate this hypothesis. L929 cells were seeded on the RGD functionalized microparticles, with encapsulated PL and cultured for 11 days. In order to study the influence of encapsulated PL in cell function, RGD functionalized microparticles without encapsulated PL were used as a control. Cell adhesion was monitored by optical microscopy. The images show an increase in cell attachment in MeLam microparticles with encapsulated PL. This may be due to the high release of proteins over the first 12h of incubation, that would enhance cell attachment. Phalloidin and DAPI staining was performed to evaluate cell morphology on the surface of high MeLam microparticles at 3, 7 and 11 days (Fig. 6A to 6G). The dependence of cell adhesion and morphology from the encapsulated PL was evident. The L929 cells adopt an elongated, spreading morphology on the microparticles containing PL and after 11 days, was possible to observe the surface of high MeLam microparticles completely covered with cells. Several cells exhibit a spindle-like morphology forming connecting points between microparticles, demonstrating that the construct provides an appropriate environment for cell proliferation. In the absence of PL, few cells adhered on the surface; this may be justified by the presence of the RGD moieties as plain microparticles did not show any cell attachment. The viability of L929 cells was assessed at day 3, 7 and 11 (Fig. 6H). In the first-time points, no significant differences between the sample and control were observed. After 11 days of culture, L929 cells exposed to PL showed significantly increase in cell viability. The results from DNA quantification corroborate the hypothesis that PL have a positive influence in cell proliferation (Fig. 6I). Cells cultured on particles with encapsulated PL have a significantly increase in DNA content after 7 days of culture. 3D assembly of the multifunctional microgels due to cell connecting points was also demonstrated.

4 Conclusions

We demonstrated an efficient one-step method to generate quasi-monodisperse MeLam microparticles incorporating PL using a microfluidics device coupled to a source of UV light. The pendant acrylate groups of MeLam allowed also the conjugation of thiolated biotin via thiol-Michael addition and further conjugation with RGD peptides. The multifunctional MeLam microparticles loaded with PL were seeded with L929 cells and the results demonstrate their potential to support cell adhesion and expansion. MeLam microgels offer a high degree of tunability over both structural and chemical properties, and can be used to recapitulate highly varied tissue environments. Cultured microgels could self-assemble to form structures with packing densities, suggesting potential applications in tissue engineering and regenerative medicine.

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Figure 1.
Schematic representation of microfluidic system used to produce MeLam microparticles.
Droplets are formed into the microfluidic chip and crosslinked with UV light.

Figure 2.

(A) Schematic illustration of the methacrylation reaction of laminarin with glycidyl methacrylate. (B) ^1H NMR spectrum of laminarin before modification. (C) Low methacrylated laminarin and (D) High methacrylated laminarin.

Figure 3.

Optical microscopy images of (A) high MeLam microparticles after UV crosslinking immersed in oil and (B) after swelling in PBS and (C) low MeLam microparticles after UV crosslinking immersed in oil and (D) after swelling in PBS. SEM image of high (E) and low (F) MeLam microparticles. Histogram of particle size distribution of (G) High MeLam and (H) Low MeLam microparticles.

Figure 4.

(A) Cumulative protein profile release from low and high methacrylate laminarin microparticles by incubation in PBS solution up to 14 days quantified by micro-BCA assay. ELISA assay performance to quantify specific growth factors release from microparticles. (B) Weight of VEGF and TGF-β1 presented in encapsulated PL. VEGF release profile (C) and TGF-β1 release profile (D) up to 14 days from low and high methacrylate laminarin. Error bars represent standard deviation (n = 3).

Figure 5.

(A) Schematic representation of the modification procedure. Microparticles are functionalized with Biotin-PEG-SH and subsequently conjugated with streptavidin and biotin-RGD to promote cell adhesion. The cultured microparticles are then capable of forming 3D robust structures. Fluorescence images showing the functionalization of MeLam microparticles. Images of MeLam microparticles with biotin-PEG-SH (B) and MeLam microparticles without biotin (control) (C) after incubation with fluorescently labeled SaV. Images of MeLam microparticles with biotin-PEG-SH bioconjugate with pure SaV (D) and without bioconjugation with pure SaV (control) (E) after incubation with fluorescence biotin.

Figure 6.

Fluorescence images of the high MeLam microparticles with encapsulated PL (A-D) and high MeLam microparticles without encapsulated PL (E-G) culture with L929 cells up to 11 days. Cytoskeleton was stained with phalloidin (red) and nuclei was stained with DAPI (blue). (E) The metabolic activity was determined by MTS assay at 3, 7 and 11 days. (F) DNA quantification of all formulations tested up to 11 days of culture. Results are present as mean $\pm$ standard error of the mean (n = 3).