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Developing hyaluronic acid microgels for sustained delivery of platelet lysate for tissue engineering applications

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ABSTRACT

Platelet lysate (PL), a blood product that contains high concentrations of growth factors (GFs), can be considered as a cost-effective source of multiple GFs. In this study, hyaluronic acid (HA) based microgels were developed for delivery of PL proteins. Spherical microgel were prepared using a water in oil emulsion method. First, hyaluronic acid was grafted with tyramine groups, after which prepared microdroplets were crosslinked via an enzymatic reaction in the presence of hydrogen peroxide and horseradish peroxidase. Because of electrostatic interactions, these microgels are promising carriers for positively charged proteins entrapment like most of the GFs. When microgels are incubated in PL solution, protein loading takes place which is mainly governed by nonspecific adsorption of plasma proteins. Although this hampered loading efficiency, loading could be increased by repeated washing and incubation steps. The loaded microgels presented a sustained release of PL growth factors for a period of two weeks. When PL enriched microgels were embedded in a HA bulk hydrogel, cell proliferation was higher compared to constructs without microgels. These findings suggest that the developed microgels are a potential candidate for sustained delivery of PL growth factors and present a solution to the issue of their short half-lives in vivo.

1. Introduction

In tissue healing processes, the main cellular activities are regulated by a combination of several growth factors (GFs) and cytokines present in the body. Although all functions of GFs in tissue regeneration are not completely known, the crucial role of many of them have been demonstrated [1–3]. Whereas the use of a single GF may achieve limited success, combinations of GFs favors tissue formation. The use of multiple growth factors for tissue regeneration, however, is hampered by their high cost. Moreover, realization of proper healing responses using combinations of GFs remains difficult [4]. A suitable and inexpensive source of multiple growth factors and bioactive agents is platelet lysate (PL) which is obtained from platelet rich plasma (PRP). PRP is a blood derivative obtained from patient's blood through a well-established procedure [5,6]. Upon activation or membrane rupture, platelets release a pool of growth factors and other bioactive agents present in their alpha granules, such as transforming growth factor (TGF-β), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and fibroblast growth factor (FGF) [7]. The presence of such a cocktail of bioactive factors may have synergistic effects leading to more effective tissue regeneration [2].

Bolus injection of growth factors may not have desired outcomes because of their short biological half-lives in vivo. Also, delivery of high doses of the proteins may induce side effects [8]. Hence, there is a need to develop suitable approaches to deliver the proteins to the desired site in a controlled manner. For this purpose, polymeric materials have shown a pivotal role in sustained, controlled, and localized delivery of proteins. Moreover, encapsulated GFs would be protected against enzymatic degradation [2,8]. Encapsulating the protein mixture in a polymeric matrix, containing several GFs as present in PL, may thus be an alternative and inexpensive method to deliver in a controlled way the GFs and to sustain their effect on tissue regeneration.

As a matrix for the delivery of PL, microgels, crosslinked hydrophilic polymer networks having a large surface area, high

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water content and tunable size, were selected [9–14]. Although nano- and microparticulate systems have been widely used for GFs delivery, only a few studies have used PL as an autologous and cost-effective source of growth factors [8,12]. Santo et al. developed chitosan-chondroitin sulfate nanoparticles to physically adsorb PL on the surface. In this way, the release of PL proteins was extended up to one week [15]. In another study, hyaluronic acid (HA) microparticles loaded with PL were incorporated in calcium phosphate cements. The release of PL proteins was detectable for up to 8 days, and the expression of osteogenic markers by human adipose stem cells was upregulated [16].

A wide range of synthetic and natural polymers have been used so far for fabricating microgels, including PEG [17], gelatin [18], hyaluronic acid [11], alginate [19], and chitosan [15]. Of those, hyaluronic acid (HA), a linear polysaccharide, is a promising biomaterial because of its excellent biocompatibility, biodegradability, and low immunological risks. Also, the presence of different functional groups like hydroxyl and carboxyl groups on the HA molecular chain provides anchors for various chemical modifications [11,20–23].

In a previous study, it was demonstrated that hMSCs could be encapsulated in in-situ forming enzymatically crosslinked tyramine conjugated hyaluronic acid (HA-TA) hydrogels and remain alive for a long period of time [24]. In this study, HA-TA based microgels were prepared to load and release PL proteins. The microgels were fabricated using a water in oil emulsion method followed by enzymatic crosslinking. Also, microgels with different crosslinking densities were produced and the effect of network crosslinking on the swelling ratio, size and protein loading was investigated. hMSCs cell viability and proliferation, by adding PL-loaded microgels to the cells directly or indirectly using transwells, were determined. Moreover, microgels and cells encapsulated in bulk hydrogels were studied for their biocompatibility. In the current work, we demonstrated that incorporating PL-loaded microgels into a HA hydrogel is a usable strategy for increasing the bioactivity of the scaffold. In this way, the triad of tissue engineering, using cells, engineered materials, and signaling molecules simultaneously may be used to create functional tissues.

2. Materials and methods

2.1. Materials

Sodium hyaluronate (MW = 25 kDa) was purchased from Contipro Pharma, the Czech Republic and used without further purification. Tyramine (TA) (99%), anhydrous N,N-dimethylformamide (DMF) (99.8%), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDAC), N-hydroxysuccinimide (NHS), hydrogen peroxide (H_2O_2), horseradish peroxidase (HRP, 325 units/mg solid), Hyaluronidase from bovine testes (lyophilized powder, 400–1000 units/mg), and MES hemisodium salt dry powder were obtained from Sigma-Aldrich and used without further purification. Phosphate buffered saline (PBS, 10 mM, pH 7.4) was purchased from Lonza. All other solvents were purchased from Biosolve (Valkenswaard, The Netherlands) and used as received. 2,2,4-Trimethylpentane (Isooctane) was purchased from Honeywell. Tween 80, Span 80, lysozyme from chicken egg white, bovine serum albumin (BSA) and immunoglobulin G (IgG) were purchased from Sigma-Aldrich. All reagents were used as received unless otherwise noted. Platelet lysate and heparin sodium salt solution (5000 U/mL) were purchased from PL BioScience (Aachen, Germany).

2.2. Macromere synthesis

Tyramine conjugated hyaluronic acid (HA-TA) with a degree of substitution of 17% as determined by ^{1}H NMR was synthesized based on a previous study [25]. Briefly, sodium hyaluronate (1.0 g) was dissolved in 30 mL of PBS and EDAC (1.0 g) and NHS (1.3 g) were added. After stirring for 2 h, the product was precipitated in the cold ethanol, filtered and dried at room temperature. The HA with NHS groups (1.0 g) was dissolved in 0.1 M MES buffer (pH 6.1). Then, 4 mL of a tyramine solution in DMF (96 mg/mL) was added to the solution and the mixture was stirred overnight, precipitated in cold ethanol and filtered. The polymer was collected, dissolved in Milli-Q water and transferred to a dialysis tube (Spectrum Labs, MW cut-off 1 kDa). The solution was purified by successively dialyzing against 100 mM sodium chloride solution, a mixture of distilled water and ethanol (5:1) and MilliQ-water, and then lyophilized.

2.3. Preparation of hyaluronic acid-tyramine microgels

HA-TA crosslinked networks could be formed by a HRP catalyzed radical crosslinking reaction using H_2O_2 and the gelation time was controlled by changing the amount of HRP and H_2O_2. Hyaluronic acid microgels were synthesized with this method using a water in oil emulsion as previously described [25]. Briefly, 2 mL of a HA-TA solution (5% w/v) containing HRP (1.5 U/mL) was added dropwise into 25 mL of ice cooled isooctane, Span 80 (2.5% v/v) and Tween 80 (0.25% v/v). The resulting mixture was homogenized using an Ultra-Turrax (T25, IKA Works Inc., USA) at 13500 rpm. To start the crosslinking process, 20 μL of a H_2O_2 solution in PBS with different concentrations was added to the microemulsion to give a H_2O_2/TA molar ratio of 0.5, 0.3 or 0.1. The reaction proceeded for 30 min to complete crosslinking. The emulsion was broken up by precipitation in ice-cold acetone and particles were collected by centrifugation at 8000 rpm for 5 min. The resulting particles were washed with acetone and ethanol to remove excess oil and dried at room temperature at reduced pressure. To visualize the microgels, they were dispersed in water and imaged using a phase contrast microscopy (Nikon Eclipse TE300 microscope equipped with Nikon Digital Sight DS-fi1 camera). The average diameter of the microgels in the swollen state was determined using Image J software. The microgels made at H_2O_2/TA molar ratios of 0.5, 0.3, and 0.1 were abbreviated H0.5, H0.3, and H0.1, respectively.

To determine the swelling ratio of microgels, 3 mg of dried microgels were placed in a 2 mL Eppendorf vial and 1 mL of PBS (10 mM, pH = 7.4) was added. The particles were dispersed using a shaker and incubated at 37 °C for 3 h. Microgels were centrifuged at 5000 rpm for 8 min and the excess water was discarded and blotted from microgel pellet and vial. The swelling is expressed as the ratio of weight in the swollen state to dry weight of microgels (W_s/W_d).

2.4. Protein encapsulation

Platelet lysate is a mixture of plasma proteins like serum albumin and IgG, and also contains different types of growth factors. In preliminary studies, lysozyme (14 kDa, pI = 11.35 [26]) was used as a model protein that resembles growth factors in charge and size, like TGF-β1 (25 kDa, pI = 8.83 [27]), PDGF-BB (24 kDa, pI = 9.8 [28]), VEGF (27 kDa, pI = 8.5 [29]), and BMP-2 (32 kDa, pI = 8.4 [30]).

In model experiments the capacity of microgels for lysozyme uptake was compared to IgG and BSA which are also present in PL. Dry microgels (2 mg) with different crosslinking densities were incubated with 1 mL of lysozyme, BSA or IgG solutions at a concentration of 500 μg/mL. After 16 h incubation, under a 100 rpm constant shaking, the microgels were centrifuged (7000 rpm, 10 min) and the supernatant was removed and analyzed for its protein content using a Bradford Assay (Coomassie protein assay kit, Thermo scientific). Unloaded microgels served as a negative control.

To prepare PL-loaded microgels, 5 mg of microgels were incubated in 1 mL of the PL overnight. Subsequent steps were performed as discussed above for single protein loading. The total amount of proteins present in the PL was determined using a Bradford assay, measuring the absorbance spectrophotometrically (Multiskan GO, Thermo Scientific) at 595 nm. The amount of TGF-β1 encapsulated was quantified by an Enzyme-Linked Immunosorbent Assay (ELISA, R&D systems).

2.5. In-situ forming hybrid hydrogels

To incorporate PL-loaded microgels in the HA-TA bulk hydrogel network, microgels were dispersed in a 5% w/v solution of HA-TA with a concentration of 10 mg dry microgels/mL. Hydrogel discs of 300 μL with a diameter of 9 mm were prepared in a mold. Hybrid hydrogels were prepared by addition of 20 μL of H_2O_2 (1.5% v/v) and 12 μL of a HRP solution in PBS (2 mg/mL, 650 U/mL). The final concentrations of H_2O_2 and HRP were 9.8 mM and 8 U/mL, respectively. To show the distribution of microgels in the hydrogel, FITC-dextran loaded microgels were used and the construct was imaged by an EVOS digital microscope (Invitrogen, Netherlands).

The mechanical properties of the hydrogel with and without microgels were determined by rheology (Anton Paar Physical NICR 301 rheometer with a 25 mm diameter spindle in the oscillatory mode). The measurements were taken at 37 °C and the initial force was kept constant at 0.5 N/m^2. For these measurements hydrogels were made in a mold with a diameter of 20 mm and height of 3 mm. The storage (G′) and loss (G″) modulus were recorded using at a strain of 1% and a frequency of 1 Hz.

2.6. In vitro protein release

PL-loaded microgels (3 mg) were placed in a 1.5 mL low protein binding collection tube (Thermo Fisher Scientific) and incubated with 1 mL of PBS (10 mM, pH = 7.4) containing 0.05% w/v sodium azide (to prevent bacterial growth) under a 100 rpm constant shaking. The release of proteins was monitored over a period of two weeks. At regular time intervals, the microgels were centrifuged (7000 rpm, 10 min), the supernatant was collected and replaced with a fresh medium. The amount of total protein released was assessed using a Bradford assay. Release samples were analyzed for TGF-β1 content via ELISA (human TGF-β1 ELISA kit) according to the manufacturer's protocol.

To compare the release profile of proteins from microgels and microgel/hydrogel hybrid scaffolds, disk-shaped constructs (9 mm in diameter and height of 3 mm) containing 3 mg of dry microgels were transferred to a tube and immersed in 1 mL of PBS containing 0.05% w/v sodium azide. At pre-determined time points, the medium was collected and replaced with an equal amount of fresh medium. The protein content in the medium was assessed as presented above.

2.7. Cell response in the presence of PL-loaded microgels

2.7.1. hMSC isolation and pre-culturing

Human bone marrow derived mesenchymal stem cells (hMSCs) were isolated as previously reported [31], and cultured in MSC proliferation medium (α-MEM (Gibco) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine (Gibco), 0.2 mM ascorbic acid (Gibco), 100 U/mL penicillin, 10 μg/mL streptomycin and 1 ng/mL bFGF). The use of human material was approved by a local medical ethical committee. All reagents used were purchased from Invitrogen (Paisley, UK) unless otherwise stated. When hMSCs reached confluence, the cells were detached using 0.25% Trypsin/EDTA (Gibco) and subsequently subcultured or used for experiments.

2.7.2. 2D cell experiments

Before cell experiments, the microgels were sterilized using 70% ethanol and dried under reduced pressure. Human MSCs at passage 4 were cultured in 24 well plates at an initial density of 1000 cells/cm^2 and allowed to attach for 12 h at 37 °C in a 5% CO_2 incubator. To see the effect of PL-loaded microgels on cell proliferation, following treatments were added to the wells: (1) medium with 10% FBS, (2) medium with 10% PL, (3) 2 mg of PL-loaded microgels in a medium with 1% FBS added on top of the cells, (4) 2 mg of PL-loaded microgels in a medium with 1% FBS added in a transwell, and (5) medium with 1% FBS as negative control. For transwell experiments, cells were cultured at the bottom of the plate and the microgels were added above the polycarbonate membrane (0.2 μm pore size). For wells containing microgels, low serum media (1% FBS in α-MEM) was used.

To investigate the effect of different concentrations of PL-loaded microgels on cell proliferation, hMSCs were cultured at the bottom of a 48 well-plate and different amounts of microgels (from 0.125 mg to 2 mg) were added on top of the cells. Then, 250 μL of low serum medium (1% FBS) was added to each well and the medium was changed every 2 or 3 days. To prevent suction of microgels during medium change, each time half of the medium was discarded and replaced with fresh one.

2.7.3. 3D cell experiments

Isolated hMSCs at passage 4 and PL-loaded microgels were encapsulated in a HA-TA bulk hydrogel. The concentration of microgels was 10 mg/mL and cells were encapsulated at a density of $2*10^6$ cells/mL. First, 1 mL of HA-TA solution (5% w/v) was added to 10 mg of sterile microgels loaded with PL. The microgels were suspended homogenously by gently pipetting up and down the solution. Next, half of the precursor gel solution was added to a cell pellet and mixed with HRP solution to get a final concentration of 16 U/mL. The other half of the precursor solution was prepared by addition of H_2O_2 (final concentration 19.6 mM). The HRP and H_2O_2 stock solutions were made using PBS. Subsequently, 40 μL hydrogels were prepared by mixing 20 μL of the solutions in a round bottom 96-well plate. After crosslinking, hydrogels were placed in a non-adherent polystyrene 48 well plate with 500 μL of hMSC culture medium without bFGF on top. The medium was exchanged every 2 or 3 days.

2.7.4. Cell metabolic activity and DNA quantification

The metabolic activity of the cells, cultured in wells, was measured using a Prestoblue assay (Invitrogen). Briefly, cells at the bottom of the wells were rinsed twice with PBS and incubated with a 10% solution of Prestoblue in culture medium for 2 h. The absorbance was measured using a microplate reader at 530 nm excitation and 590 nm emission.

The DNA content of the cells cultured in 2D and the cell laden hydrogel constructs was assessed via a CyQuant DNA Assay kit according the manufacturer's description (Molecular Probes, Eugene, Oregon, USA), and using a fluorescent plate reader (excitation: 480 nm; emission: 520 nm) (Perkin-Elmer, Victor 3, USA). For determining the DNA content of the cells cultured in 2 D, 250 μL of lysis buffer (0.1 M KH2PO4, 0.1 M K2HPO4, and 0.1% v/v Triton X-100, pH 7.8) was added to each well and incubated for one hour at room temperature. Cell laden hydrogel constructs were transferred to 2 mL Eppendorf vials and 1 mL of lysis buffer was added to each sample, homogenized, and incubated for one hour. The standard curve for DNA analysis was generated with λ DNA included in the kit.

Viability of the encapsulated cells in HA-TA hydrogel was assayed by a Live/dead kit at day 1 and 7. The hydrogel constructs were rinsed twice with PBS and then stained with calcein AM/ethidium homodimer using the Live-dead assay Kit (Invitrogen)

according to the manufacturer's instructions. The cells in hydrogels were visualized using a fluorescence microscope (EVOS). Living cells fluoresce green and the nuclei of dead cells red.

2.8. Statistical analysis:

Each experiment was performed in triplicate. The results are presented as the mean ± standard deviation (SD). Experimental data were analyzed for statistical significance using a one-way analysis of variance (ANOVA) with Turkey's post hoc analysis. Statistical significance was set to *p-value <0.05, **p-value <0.01, and ***p-value <0.001.

3. Results and discussion

3.1. Preparation of HA-TA microgels

Tyramine was conjugated to the HA successfully in a two-step procedure as shown by ^{1}H NMR [25]. HA-TA microgels were fabricated by addition of H_2O_2 to a microemulsion of HA-TA containing HRP in isooctane as schematically shown in Fig. 1A. Washing and isolation of the microgels afforded dry microgels as a fine powder (Fig. 1B), which could be easily dispersed in water. Microgels with different crosslinking densities were prepared by changing the H_2O_2/tyramine molar ratio. Microscopic images of the microgels and

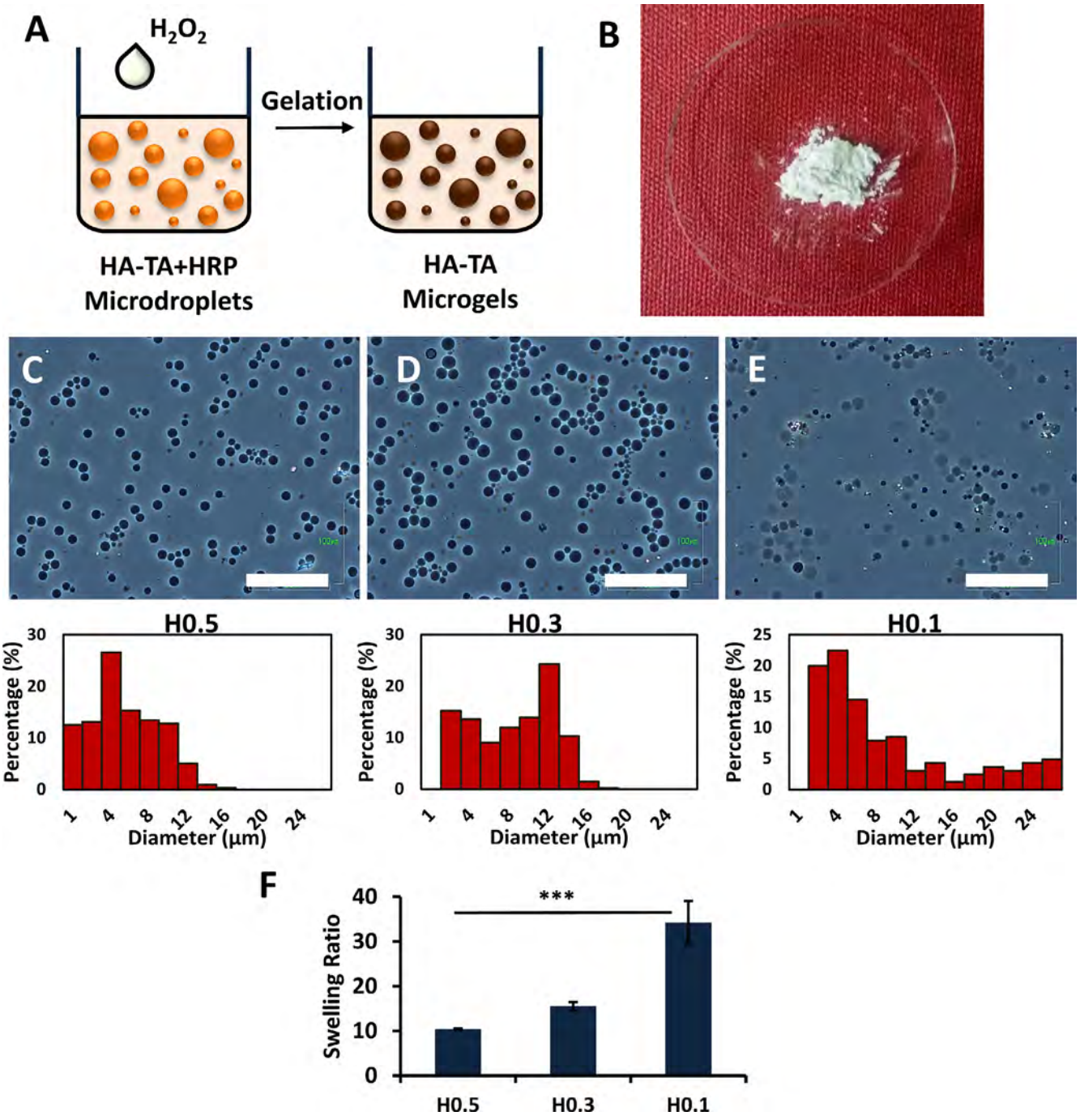

Fig. 1. (A) Schematic representation of the preparation of HA-TA microgels by a water in oil emulsion method. (B) Macroscopic appearance of dry microgels. Light microscope images and size distribution of HA-TA microgels prepared at a H_2O_2/TA molar ratio of 0.5 (C), 0.3 (D), and 0.1 (E). Scale bars are 100 μm. (F) Swelling ratios of H0.5, H0.3 and H0.1 microgels at 37 °C. Error bars are means ± SD (n = 3) and ***p < 0.001.

their size distribution analyzed with Image J software are depicted in Fig. 1C–E. Microscopic images of the microgels revealed increasing average diameters of 4.6 ± 3.1, 7.3 ± 4.0, and 8.2 ± 7.9 μm for microgels prepared at decreasing H_2O_2/TA molar ratios (H0.5, H0.3, and H0.1). By lowering the amount of H_2O_2 not only large microgels appeared, but also the dispersity became very broad likely caused by a decrease in the crosslinking density. This is in accordance with previous studies showing that with increasing crosslink density the relative swelling of HA-TA hydrogels decreases [24].

The equilibrium swelling ratio of microgels is presented in Fig. 1F. As shown, the swelling ratio of H0.5 microgels is significantly lower than that of H0.1 because of a more densely crosslinked network.

The presented HA-based microgels degrade through hydrolysis as shown in a previous study [25]. The general trend observed was an increase in water content as a result of network cleavage and complete dissolution after four weeks.

3.2. Protein loading capacity of HA-TA microgels

In model experiments the protein uptake efficiency of the HA-TA microgels was determined using lysozyme, BSA, and IgG. Lysozyme could resemble the growth factors present in PL in charge and size, and serum albumin and IgG are the main soluble proteins in plasma. As depicted in Fig. 2A, lysozyme was encapsulated in the H0.5 and H0.3 microgels with a good efficiency (~60%). Interestingly, the loading efficiencies of much larger proteins like BSA and IgG were substantially lower; 17% and 14% for H0.3 and ~6% and 7% for H0.5 microgels, respectively. The differences in loading likely relates to the charge and size of the proteins and the crosslinking density of HA-TA microgels. Lysozyme is a positively charged protein at pH 7.4 with an isoelectric point of 11.35, while HA is a negatively charged polymer (p*I* = 2.5 [32]). Therefore, lysozyme can electrostatically bind to HA-TA microgels. Conversely, BSA has an acidic isoelectric point of 4.7 [33] and is expected to be adsorbed non-specifically and therefore easily washed from the surface of

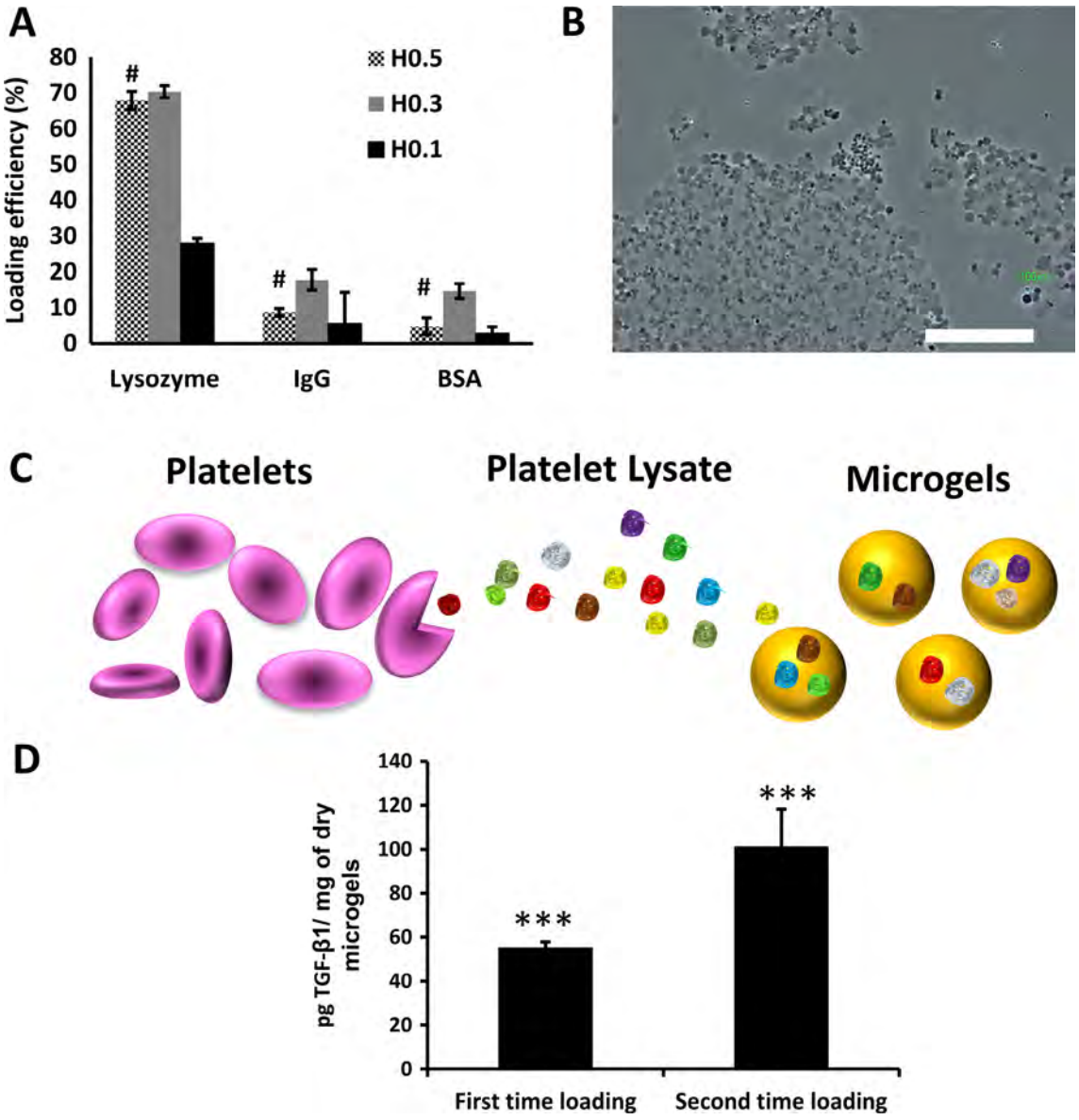

Fig. 2. (A) Lysozyme, IgG, and BSA loading capacity of HA-TA microgels. (B) Agglomeration of microgels prepared at a H_2O_2/TA molar ratio of 0.1 after one day. (C) Schematic representation of PL loading in HA-TA microgels. (D) Loading of TGF-β1 into HA-TA microgels from a PL solution. Error bars were means ± SD (n = 3; *** and #p < 0.001).

the microgels. Although IgG is a neutral protein at physiological conditions, its loading efficiency is as low as BSA. The reason is possibly due to the protein size, 2.5 times larger than BSA [34].

There were no significant differences between the lysozyme uptake of H0.3 and H0.5 microgels. However, H0.1 microgels showed a very low capacity for lysozyme loading. The reason could be the agglomeration of the microgels due to incomplete crosslinking at the low H_2O_2/TA molar ratio (Fig. 2B). These results decided us to select the H0.5 microgels in further studies towards a potential carrier system based on PL, which releases growth factors.

In following experiments, H0.5 microgels (5 mg) were incubated with 1 mL of PL overnight. The loading of PL proteins in microgels is schematically shown in Fig. 2C. To assess the amount of TGF-β1 which was present in the PL loaded microgels, the supernatant was removed and replaced with 1 mL of hyaluronidase solution (200 U/mL). The microgels were completely degraded after overnight incubation at 37 °C. The amount of TGF-β1 in the solution was measured by ELISA and the results are shown in Fig. 2D. The concentration of TGF-β1 in the PL solution used in this study was approximately 70 ng/mL. The total amount of TGF-β1 loaded in the microgels was 277 pg (55.4 pg/mg dry microgels) which was much lower than the initial amount of TGF-β1 present in PL (Fig. 2D). This low loading efficiency may be caused by the relatively large amounts of plasma proteins like serum albumin and IgG. These proteins, although less efficiently taken up (vide supra) may cover the surface of the microgels and hinder the uptake of growth factors. These loaded microgels were therefore washed one time with PBS and again incubated with 1 mL of PL. In this way, the amount of loaded TGF-β1 increased to ~506 pg (101.2 pg/mg dry microgels) (Fig. 2D). To determine the capacity of HA-TA microgels for growth factor loading without the presence of plasma proteins, the microgels were incubated with 100 ng TGF-β1 in 1 mL of PBS which afforded an uptake of 97%. It can be concluded that HA-TA microgels have a high loading capacity of cationic proteins like growth factors present in PL, but the presence of plasma proteins in relative high concentrations interfere with their binding. Decreasing the concentration of albumin and IgG in PL with respect to the growth factors may be an effective strategy to increase the loading efficiency of most of the PL growth factors.

3.3. Hybrid hydrogels

Microgels can be utilized alone or in combination with three-dimensional hydrogel structures (hybrid hydrogels) [35–37]. To efficiently prepare such hybrid hydrogels the distribution of the microgels in a bulk HA-TA hydrogel was tested. To this extend FITC-dextran loaded microgels were mixed with a HA-TA solution which was subsequently enzymatically crosslinked using HRP and H_2O_2 (Fig. 3A). Results showed that the microgels could be homogeneously distributed in the bulk hydrogel (Fig. 3B). The storage modulus (Ǵ) and loss modulus (G′) of standard sized HA-TA hydrogels (20 mm in diameter and 3 mm thickness), prepared at a H_2O_2/TA molar ratio of 0.5, were determined at 37 °C by oscillatory rheology. Hydrogels with and without HA-TA microgels showed G′ values of 5400 and 3700 Pa, respectively (Fig. 3C). The G″ values of hydrogels with microgels, however, were slightly higher which may be due to a somewhat lower crosslinking efficiency.

3.4. In vitro release of PL proteins

The release profiles of PL proteins from H0.5 microgels and from microgel/hydrogel constructs during 14 days were determined and

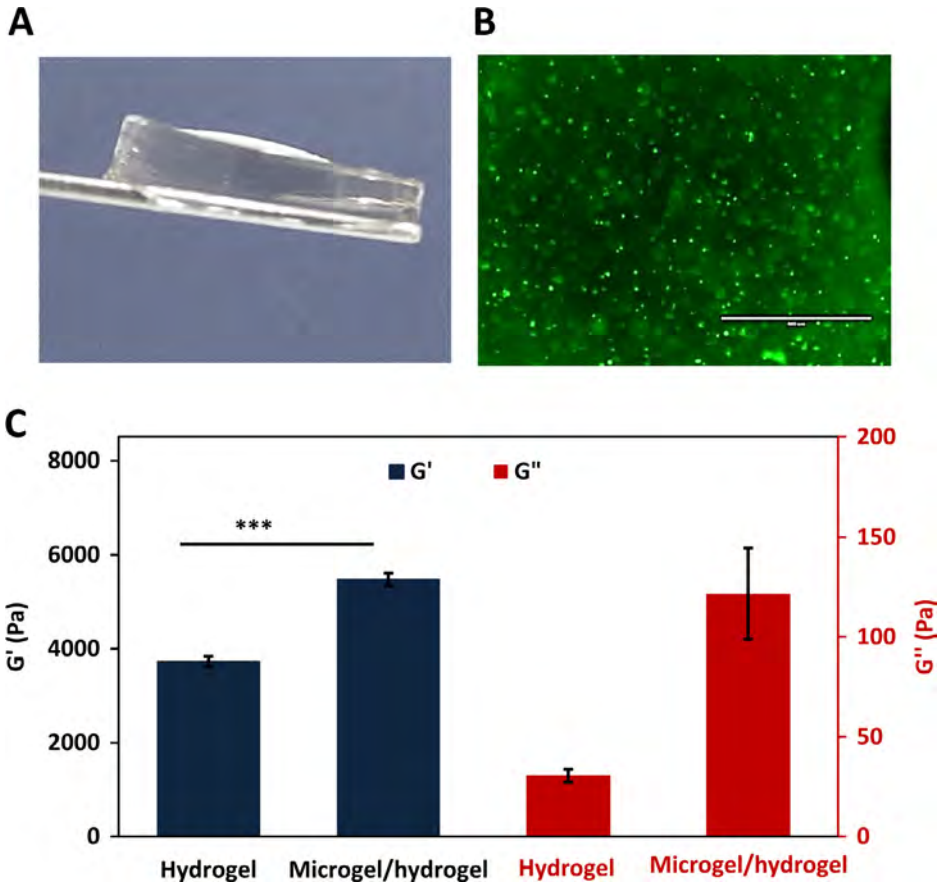

Fig. 3. (A) Macroscopic appearance of a HA-TA hydrogel. (B) Uniform distribution of HA-TA microgels loaded with FITC-dextran and incorporated in a HA-TA bulk hydrogel, scale bar is 400 μm. (C) Storage (G′) and loss moduli (G″) of HA-TA hydrogels with and without incorporated microgels.

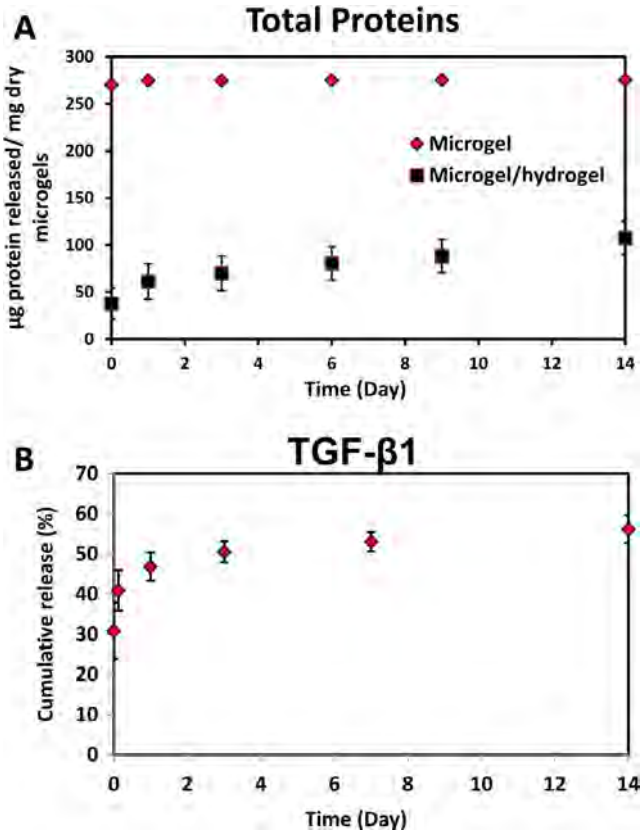

Fig. 4. In vitro PL-proteins release profiles. (A) Total protein release measured by a Bradford assay from microgels and microgel/hydrogel constructs. (B) The release of TGF-β1 from PL-loaded microgels. Error bars are means ± SD (n = 3).

the results are depicted in Fig. 4. The released amount of total proteins and TGF-β1 were analyzed by a Bradford assay and ELISA, respectively. As shown in Fig. 4A, proteins were released from PL-loaded microgels during the initial stages of incubation. This was expected because albumin and IgG, which represent 79 ± 1% of PL proteins, [38] were adsorbed non-specifically to the negatively charged HA-TA microgels, and consequently washed out rapidly. However, when an equal amount of PL-loaded microgels were embedded in HA-TA hydrogels slower and sustained release was achieved and this burst effect was largely reduced.

The cumulative TGF-β1 release profile from the microgels showed a fast release up to 40% during the first 7 h followed by a more sustained release (Fig. 4B). Remarkably, the release of TGF-β1 from the PL loaded microgels in the hybrid hydrogels was not detectable showing the entrapment of the protein in the structure of the hydrogel. Hence, the results show the capability of HA-TA microgels for growth factor release which can be further modulated by incorporating the PL loaded microgels in a bulk hydrogel.

3.5. Effect of platelet lysate released from microgels on cell biological activities

Human platelet lysate has previously been introduced as an alternative to FBS in cell culturing [38,39]. The applied human PL was therefore first tested on cell proliferation. Human MSCs were cultured in a medium supplemented with 10% FBS or 10% PL, and clearly, cells were more confluent in PL media after 3 days of culture (Fig. 5 A and B). To show if the PL-loaded microgels provide an anticipated enhancement of cell proliferation, cells were seeded at the bottom of a 24 well plate and PL-loaded microgels were placed on top of the cells (direct contact) or above using a transwell (Fig. 5C). Fig. 5D shows the hMSCs cultured in direct contact with microgels after three days.

The proliferation of cells in the wells supplemented with PL-loaded microgels, in comparison to the negative control (medium with 1% FBS), was enhanced (Fig. 6A). Non-loaded microgels did not have any effect on cell proliferation. The proliferation rate of the cells was higher when the loaded microgels were in direct contact with the cells compared to microgels separated via a transwell

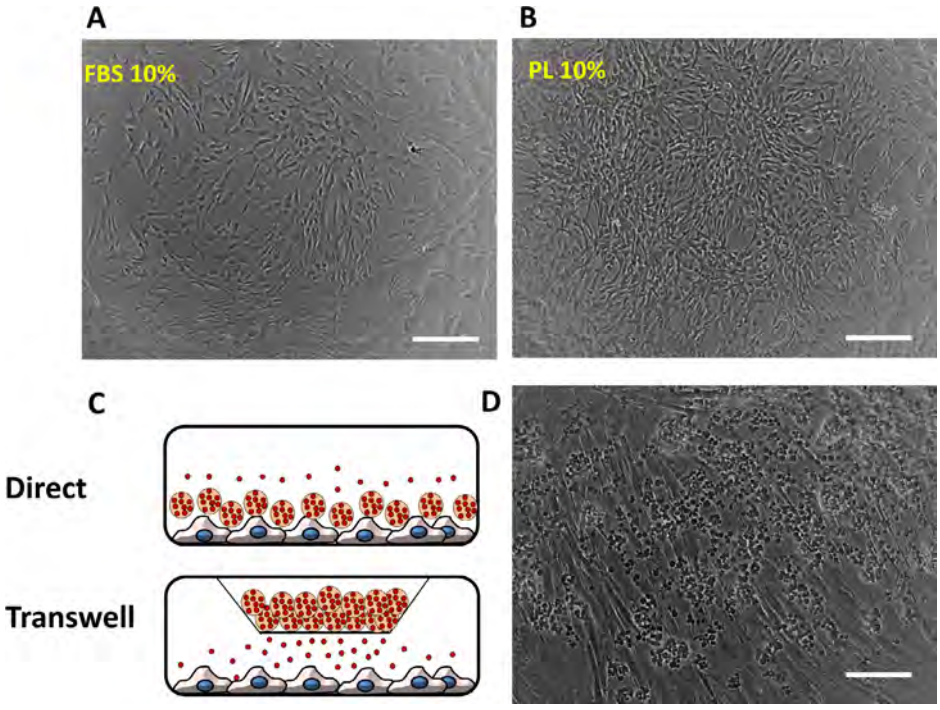

Fig. 5. Representative images of hMSCs cultured for 3 days in medium supplemented with (A) 10% FBS or (B) 10% PL. (C) Schematic representation of experiments using PL-loaded microgels and cells, in direct contact or separated by a trans-well. (D) Microscopic image of cells cultured with microgels in direct contact. Scale bars are 100 µm.

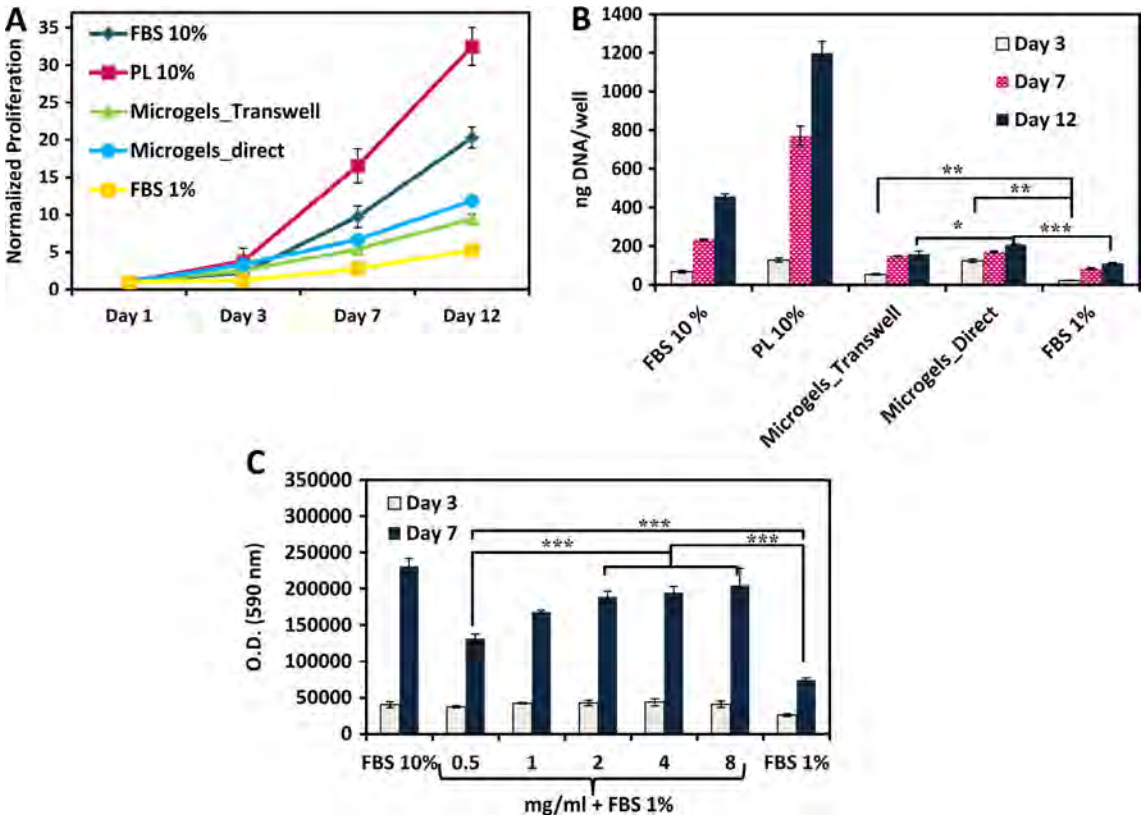

Fig. 6. (A) Response of hMSCs to PL-loaded microgels in a 2D experiment. Proliferation rate of hMSCs cultured in medium with 10% FBS, 10% PL, PL-loaded microgels in medium with 1% FBS, and low serum media (1% FBS). (B) DNA content of cells cultured at different conditions. (C) Metabolic activity of hMSCs cultured in direct contact with PL-loaded microgels at various concentrations (0.5–8 mg/mL). Error bars were means ± SD (n = 3; *p < 0.05, **p < 0.01, and ***p < 0.001).

insert. However, the effect of the PL proteins released from microgels in the transwell was clearly observed in comparison to the negative control.

Cell proliferation was confirmed by measuring the total DNA content (Fig. 7B). The total amount of DNA of cells treated with PL-loaded microgels at every time point was apparently higher than the negative control (low serum media). Cells cultured in direct contact with loaded microgels exhibited a higher DNA content compared to cells cultured with microgels separated by a transwell. In general, in stagnant environments a concentration boundary layer is formed that may act as a barrier for solute transfer [40,41]. When microgels are located above the membrane the formation of this boundary layer may reduce access to growth factors which likely explains the reduced proliferation.

Significant differences were observed between the metabolic activities of hMSCs treated with different concentrations of PL-loaded microgels in direct contact (Fig. 6C). Even a low concentration of microgels (0.5 mg/mL) had a positive effect on cell proliferation compared to the negative control. Furthermore, cell proliferation was promoted when using higher amounts of PL-loaded microgels. The ability of PL to enhance cell proliferation is in line with earlier studies [42,43].

It was shown in a previous study [24] that injectable HA-TA hydrogels can act as a carrier for cell delivery because hyaluronic acid is biocompatible and the enzymatic crosslinking is performed under mild conditions. Incorporation of PL in the bulk hydrogel increased cell attachment and promoted chondrogenesis. In following experiments, PL proteins were loaded in the microgels to act as a PL protein delivery system and together with hMSCs encapsulated in HA-TA hydrogels as schematically depicted in Fig. 7A. The viability of the encapsulated hMSCs as determined using a Live/Dead assay (Fig. 7B) was high (>95%) in all samples at days 1 and 7.

Fig. 7C shows the DNA content of each hydrogel over time. The DNA content in bulk hydrogels without loaded microgels decreased somewhat in time but was restored in hydrogels loaded with PL loaded microgels. The effects of the presence of different growth factors in PL has been reported in previous studies [44,45]. It may be concluded that the hydrogel constructs loaded with PL-loaded microgels show bioactivity to a certain extend. Besides that, microgels could protect the cargo in the process of scaffold fabrication and thus the growth factors would be less susceptible to loss in activity.

The GFs present in PL are known to have effects on wound healing [1], chondrogenesis [46], and osteogenesis [47]. Also, PL growth factors promote chemotactic migration of mesenchymal stem cells [44]. Therefore, combining PL with biomaterials to maintain the beneficial effects of PL over a longer period of time is of interest and has received more attention recently [46,48,49]. However, the PL content depends on its preparation method, activation, initial platelet concentration, and donor [50–53].

4. Conclusions

In this study, HA-TA microgels were prepared by enzymatic crosslinking of HA-TA microdroplets in the presence of HRP enzyme and H_2O_2. The resulting microgels can be dispersed easily in the aqueous phase, and are suitable for uptake of positively charged proteins and growth factors such as present in PL. HA-TA microgels are able to load platelet lysate and release the growth factors as demonstrated for TGF-β1. By incorporating the PL-

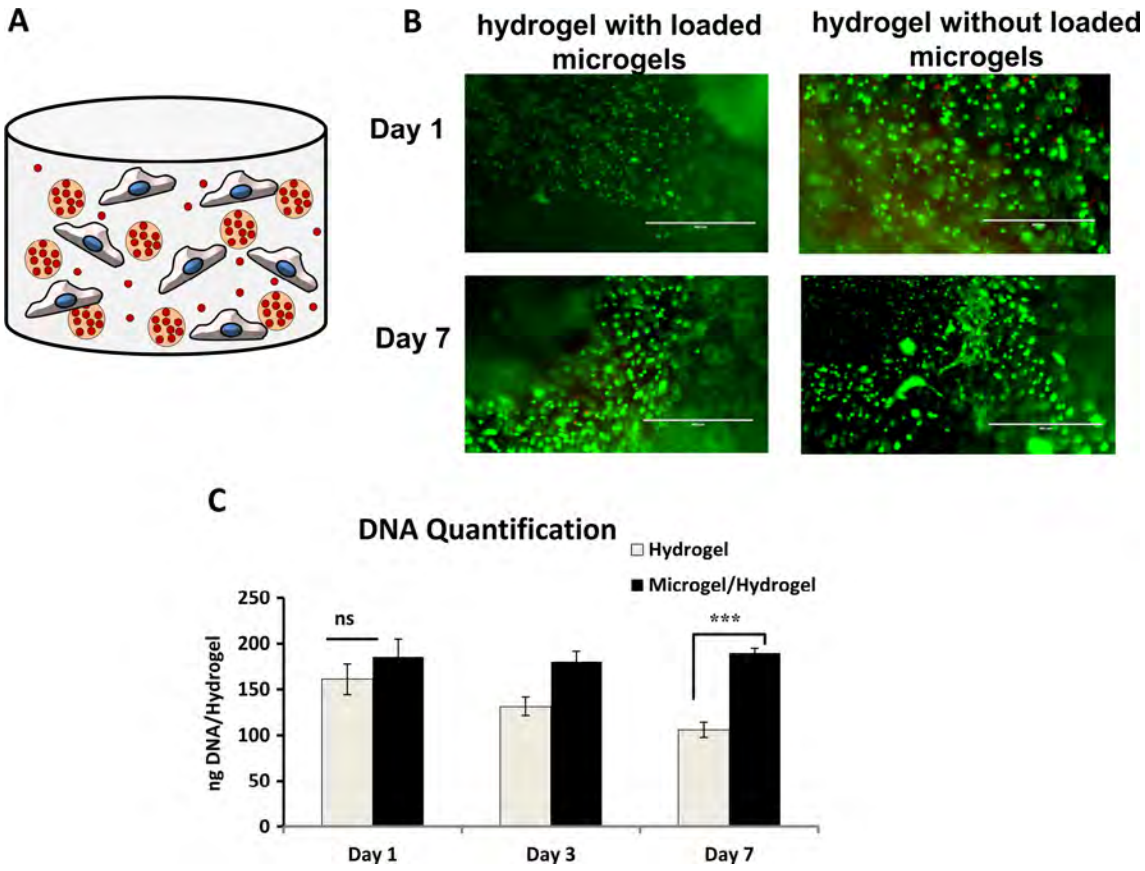

Fig. 7. Cell response to PL-loaded microgels in 3D experiments. (A) Schematic representation of a HA-TA hydrogel containing cells and loaded microgels. (B) Live/dead staining of hMSCs encapsulated in HA-TA hydrogels with and without PL-loaded microgels at days 1 and 7, scale bars are 400 μm. (C) DNA content of encapsulated hMSCs in hydrogel constructs in time. Error bars were means ± SD (n = 3; ***p < 0.001; ns: not significant).

loaded microgels in an HA-TA bulk hydrogel with hMSCs revealed a higher proliferation. As shown, by consecutive incubation with PL and washing, the loading of GFs was improved, mainly as result of removal of the non-specific adsorption of large proteins. This may be further increased by using heparin tyramine conjugates in the preparation of microgels. The proposed HA-TA microgels and microgel/hydrogel constructs are injectable at tissue defect sites to deliver platelet lysate GFs and hold high clinical potential for various applications in tissue regeneration.

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Declaration of Competing Interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2019.10.036.

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