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Effects of bound versus soluble pentosan polysulphate in PEG/HA-based hydrogels tailored for intervertebral disc regeneration

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ABSTRACT

Previous reports in the literature investigating chondrogenesis in mesenchymal progenitor cell (MPC) cultures have confirmed the chondro-inductive potential of pentosan polysulphate (PPS), a highly sulphated semi-synthetic polysaccharide, when added as a soluble component to culture media under standard aggregate-assay conditions or to poly(ethylene glycol)/hyaluronic acid (PEG/HA)-based hydrogels, even in the absence of inductive factors (e.g. TGFβ). In this present study, we aimed to assess whether a 'bound' PPS would have greater activity and availability over a soluble PPS, as a media additive or when incorporated into PEG/HA-based hydrogels. We achieved this by covalently pre-binding the PPS to the HA component of the gel (forming a new molecule, HA-PPS). We firstly investigated the activity of HA-PPS compared to free PPS, when added as a soluble factor to culture media. Cell proliferation, as determined by CCK8 and EdU assay, was decreased in the presence of either bound or free PPS whilst chondrogenic differentiation, as determined by DMMB assay and histology, was enhanced. In all cases, the effect of the bound PPS (HA-PPS) was more potent than that of the unbound form. These results alone suggest wider applications for this new molecule, either as a culture supplement or as a coating for scaffolds targeted at chondrogenic differentiation or maturation. We then investigated the incorporation of HA-PPS into a PEG/HA-based hydrogel system, by simply substituting some of the HA for HA-PPS. Rheological testing confirmed that incorporation of either HA-PPS or PPS did not significantly affect gelation kinetics, final hydrogel modulus or degradation rate but had a small, but significant, effect on swelling. When encapsulated in the hydrogels, MPCs retained good viability and rapidly adopted a rounded morphology. Histological analysis of both GAG and collagen deposition after 21 days showed that the incorporation of the bound-PPS into the hydrogel resulted in increased matrix formation when compared to the addition of soluble PPS to the hydrogel, or the hydrogel alone. We believe that this new generation injectable, degradable hydrogel, incorporating now a covalently bound-PPS, when combined with MPCs, has the potential to assist cartilage regeneration in a multitude of therapeutic targets, including for intervertebral disc (IVD) degeneration.

1. Introduction

Hydrogels are networks of hydrophilic polymers with properties that emulate those of biological tissue and are therefore promising materials for use in a wide range of tissue engineering applications. Their porous and highly hydrated nature allows for good diffusion of oxygen and nutrients whilst the three-dimensional structure provides an ideal environment for encapsulated cells. Moreover, hydrogels can be loaded with bioactive molecules, such as growth factors or drugs, to promote cell viability and direct differentiation towards a particular tissue type. For these reasons hydrogels have attracted great interest across a broad range

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of regenerative medicine applications from bone and cartilage regeneration [1,2] to cardiac tissue engineering [3].

Hydrogels are of particular relevance for the treatment of intervertebral disc (IVD) degeneration, as their properties are naturally similar to those of the nucleus pulposus (NP), a gelatinous tissue whose degradation is commonly the source of IVD disease. The NP lies at the centre of the IVD, is rich in both proteoglycans and type-II-collagen and is very highly hydrated (70–90%) [4]. Degeneration of the IVD is typically characterized by a degradation of this matrix and a resulting decrease in disc height. This reduces the ability of the disc to function in cushioning the mechanical forces that are transmitted through the spine, causing pain and leading to further degeneration. At present, there is no effective treatment to fully restore disc function. As such, hydrogel-based systems which can direct regeneration of the NP tissue and maintain disc height are attracting significant attention [5–8]. Furthermore, hydrogels can be injected into the body where they crosslink *in situ*, and mould precisely to even irregular defect spaces, eliminating the need for invasive surgical procedures. Taken together, these factors make hydrogels an exciting prospect to address the issue of IVD degeneration.

We have previously developed an injectable hydrogel system based on the enzyme-mediated crosslinking of Hyaluronic acid (HA) and poly(ethylene glycol) (PEG) that has characteristics suitable for NP regeneration [9]. Within these hydrogels we encapsulated mesenchymal precursor cells (MPCs), a purified subset of the bone marrow-derived mesenchymal stem cell population that express high levels of STRO-1, STRO-3, STRO-4, CD146 (MUC-18) and CD106 (VCAM-1). These cells have been shown to have potent regenerative potential [10–12], including for intervertebral disc regeneration [12]. When encapsulated in the PEG/HA hydrogels, the MPCs were viable for extended periods of time and showed evidence of differentiation into chondrocyte-like cells capable of synthesizing extracellular matrix components commonly found in the NP. This was particularly true for MPC/hydrogel composites that included the drug pentosan polysulphate (PPS), a highly sulphated semi-synthetic polysaccharide that has been shown to promote chondrogenic differentiation of MPCs [13] and has been used clinically for the treatment of osteoarthritis [14].

Although little is currently known about the mechanisms by which PPS exerts its effects upon MPCs, the similarities of its structure to naturally occurring sulphated glycosaminoglycans (GAGs), such as heparin and chondroitin sulphate suggest that it may play a role in regulating the presentation, stability and availability of endogenous growth factors [15], and indeed, there is some evidence to indicate interactions of this kind [16]. Both GAGs and synthetic GAG analogues [17] have been shown to promote both the osteo- and chondrogenic activity of mesenchymal populations [18–21] and many ECM-derived hydrogels contain GAGs, albeit in an undefined state. *In vivo*, GAGs are immobilized by their interactions with other ECM molecules and the covalent binding of GAGs into hydrogels to mimic this has already yielded some promising results, for example in the delivery of TGFβ or bFGF [22,23]. We therefore hypothesized that the effects of PPS upon MPC differentiation may be enhanced by covalently linking PPS into the hydrogel matrix. Here, we describe two different reaction schemes for covalent binding of PPS to the HA component of our hydrogel system and compare the effects of this new molecule upon MPC growth and differentiation to that of free PPS in solution. Finally we characterise the mechanical properties of HA/PEG hydrogels incorporating the tethered PPS and compare the properties of MPC/hydrogel composites containing either bound or free PPS.

2. Materials and methods

2.1. Materials

Hyaluronic acid of 215 kDa molecular weight was purchased from Lifecore. Amine terminated 8-arm poly(ethylene glycol) (PEG, tripentaerythritol backbone, $Mw = 40$ kDa) was purchased from JenKem, USA. 3-(4-hydroxyphenyl)propionic acid (HPA), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), sulpho-N-hydroxysuccinimide (NHS), peroxidase, Type I from horseradish (HRP, 113 U/mg solid) were all purchased from Sigma. Sodium PPS (Batch Q18) was supplied by bene-Arzneimittel GmbH (Munich, Germany). All other reagents were purchased from Gibco unless otherwise stated.

2.2. Chemistry

2.2.1. Functionalisation of HA and PEG to facilitate crosslinking into a hydrogel

HA was functionalized with tyramine (HA_{TYR}) and PEG was functionalized with 3-4-hydroxyphenylpropionic acid (PEG_{HPA}) to facilitate crosslinking using with horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2). This was done according to the chemistry established by Darr and Calabro [24] and as described by Frith et al. [9].

2.2.2. Functionalisation of HA_{TYR} with amine groups for PPS coupling

HA_{TYR} was functionalised with amine groups using cystamine hydrochloride and EDC/NHS using a 1:10:10 M ratio of acid groups on HA:cystamine hydrochloride:EDC. The reagents were dissolved in 0.1 M MES buffer, pH 4.8 and reacted for 3 h at room temperature with gentle agitation. Samples were dialysed in 3500 MWCO tubing for 48 h each against 150 mM NaCl, 10% ethanol and distilled water prior to freeze-drying.

2.2.3. Carboxylation of PPS

PPS was dissolved in 1 M chloroacetic acid, an equal volume of 3 M sodium hydroxide added and reacted at room temperature for 70 min with gentle agitation. The reaction was stopped by the addition of 4 mg/ml sodium dihydrogen phosphate and neutralised with 6N hydrochloric acid. Un-reacted products were removed by dialysis against water in 10 kDa dialysis tubing, with regular changes over a 48 h period. The solution was freeze-dried to obtain carboxylated PPS (PPS_{COOH}).

2.2.4. Coupling of HA with PPS or PPScooh

A solution of $HA_{TYR-CYS}$ and either PPS or PPS_{COOH} and EDC/NHS in a 1:5:5 M ratio was made up in 0.1 M MES buffer, pH 4.8 and reacted at room temperature, with gentle agitation for 2 h. Samples were dialysed in 10,000 MWCO tubing for 48 h each against 150 mM NaCl, 10% ethanol and distilled water prior to freeze-drying.

2.3. Charaterisation of materials

2.3.1. TNBSA assay

The degree of amine substitution of HA was measured by 2,4,6-trinitrobenzene sulfonic acid (TNBSA) assay (Thermo Scientific, USA). Briefly, 100 μl of 200 μg/ml sample in carbonate buffer (2:1, vol:vol of 0.2 M Na2CO3 to 0.2 M NaHCO3, pH 10.2) was plated out and 50 ul 0.01% TNBSA added. Samples were run in triplicate and the degree of substitution determined by comparison to a standard curve produced using glycine.

2.3.2. Fourier transform infra red (FTIR)

Samples were analysed by Fourier transform infra red (ATR-FTIR) using a Thermo Scientific Nicolet 5700 spectrophotometer, equipped with an attenuated total reflectance (ATR) module (Smart Omni sampler, GE crystal). Spectra were collected in the mid-IR range (500–4000 cm^{-1}), at a resolution of 6.0 cm^{-1} and signal averaged over 128 scans.

2.3.3. Nuclear magnetic resonance (NMR)

Quantitative 1H NMR (750 MHz) spectra were acquired on a Bruker Avance 750 high-resolution NMR spectrometer. The chemical shifts were referenced to the solvent resonance (D_2O) at $\delta = 4.77$ ppm. A comparison of the relative integrals was made between the anomeric proton of the PPS ($\delta = 5.2$ ppm) and the methyl proton of HA ($\delta = 1.9$ ppm) to determine the degree of PPS substitution. Pulsed gradient diffusion NMR was also performed on these samples to observe and approximate the amount of bound macromer, against the normalized HA methyl peak.

2.4. Characterization of hydrogels

2.4.1. Rheology

All rheological measurements were obtained using an AR G2 rheometer (TA Instruments, New Castle, DE) in oscillatory mode using a 20 mm diameter stainless steel flat plate and lower Teflon Pelletier surface. The gels were synthesized and, upon addition of the H_2O_2, immediately vortexed and loaded between the plates of the rheometer with a gap measurement of 1000 μm. Gelation kinetics and final moduli were determined by time sweeps at 37 °C, conducted with a controlled strain of 1% and an angular frequency of 6.28 rad/s. Frequency sweeps were

performed between 5.000E-3 and 50 rad/s at 1% strain and stress sweeps performed from 1 to 5 kPa at 50 rad/s.

2.4.2. Swelling and degradation

To determine the degree of swelling, 50 μl gels were synthesized and the dry weight obtained after freeze-drying for 24 h (W_d). The gels were then incubated in 500 μl PBS for 24 h at 37 °C. The PBS was removed and the gels re-weighed (W_s). The degree of swelling was determined by $((W_s - W_d)/W_d)$ using triplicate gels for each condition.

Degradation of 50 μl gels was measured over a period of 3 months using triplicate samples for each condition. After synthesis, the gels were incubated in PBS/0.01% Sodium azide at 37 °C with weekly changes of buffer. At specific timepoints all excess PBS was removed and the gels weighed. The degree of degradation was expressed as a % of the original gel mass.

2.5. MPC culture

STRO-1-selected human MPCs were prepared by Lonza (Walkersville, USA) for Mesoblast Ltd (Melbourne, Australia) according to the isolation procedure described by Gronthos et al. [25]. MPCs were cultured in alphaMEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco/Invitrogen Carlsbad, CA, USA), 10% batch-tested foetal bovine serum (FBS), 2 mM L-Glutamine, 1 mM Sodium Pyruvate, and 100 μM L-ascorbate-2-Phosphate. Tissue culture flasks were maintained at 37 °C in 5% CO_2 in an atmosphere with 95% humidity. Upon reaching 70% confluence MPCs were passaged, replating at 2000 cells/cm².

2.6. Effects of HA-PPS on growth and viability of MPCs

2.6.1. CCK8 assay

MPCs were plated at a density of 2000 cells/cm² and allowed to adhere for 24 h prior to supplementation with varying concentrations of PPS, HA-PPS or HA-PPS$_{COOH}$. After 5 days the media was removed and the cells treated with 10% CCK8 in MPC media (no phenol red) for 1 h at 37 °C. Absorbance at 450 nm was measured using a Spectromax spectrophotometer. All samples were run in quadruplicate with data for 2 independent donors pooled together.

2.6.2. EdU assay

MPCs were plated at a density of 10,000/cm² in 6-well plates and synchronised overnight in medium containing 0.5% FBS. The cells were treated for 24 h with MPC medium containing PPS, HA-PPS or HA-PPS$_{COOH}$ and 20 mM EdU solution added for a further 24 h. Cells were collected into a suspension and fixed with 4% paraformaldehyde. Determination of EdU incorporation was performed using the Click-It EdU Flow cytometry assay kit (Invitrogen) according to the manufacturer's instructions. Cells were analysed by flow cytometry using a BD LSR II Analyser to determine the proportion of the population that had undergone cell division during the 24 h treatment period.

2.6.3. qPCR

Total RNA was extracted from MPC cultures using an RNeasy Minikit with on-column DNase treatment (Qiagen) according to the manufacturer's instructions. cDNA was synthesized from using 500 ng RNA the Superscript III First strand kit (Qiagen) substituting DNase and RNase-free water for enzyme in no-RT controls. qPCR reactions were set-up in a total volume of 10 μl with 1× Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and 0.2 μM forward and reverse primers. A 7500 Fast Real-Time PCR System (Applied Biosystems) with fast cycling parameters of 2 min at 50 °C, 2 min at 95 °C then 40 cycles of 3 s at 95 °C and 30 s at 60 °C followed by a melt curve was used to run the samples. Data was analysed using the $2^{-\Delta\Delta ct}$ method.

2.7. Effects of HA-PPS on chondrogenic differentiation of MPCs

Chondrogenic differentiation was compared for MPCs cultured in maintenance medium and also in maintenance medium supplemented with PPS, HA, HA-PPS and HA-PPS$_{COOH}$ at concentrations equivalent to 5, 10 and 20 μg/ml unbound PPS. These factors were added to the cells prior to pellet formation, which was initiated by centrifuging 2×10^5 MPCs in a non-adherent U-bottom plate at 500 g for 10 min. MPC pellets were cultured in 200 μl medium with media changes every 3–4 days. After 21 days, samples were taken for analysis by histological staining, DMMB assay and qPCR.

2.7.1. DMMB assay

Chondrogenic pellets were collected, rinsed in PBS and lysed in 1 mg/ml Papain in 100 mM sodium phosphate, 5 mM EDTA and 5 mM L-cystein (pH 6.5) overnight at 60 °C. Samples of 40 μl were plated in triplicate with the addition of 10 μl 1%BSA in PBS and 50 μl 2× 1,9-dimethylmethylene blue (DMMB). These were incubated at room temperature for 15 min and the absorbance at 525 nm read using a Spectramax M5 Fluorometer (Molecular Devices). Samples were normalized to DNA content as assessed by PicoGreen assay (Molecular Probes: performed according to the manufacturer's instructions).

2.7.2. Cryosectioning and histological staining of chondrogenic pellets

Samples were fixed in 4% paraformaldehyde for 20 min, rinsed with PBS and incubated in a 1:1 mixture of OCT:30% sucrose for 4 h followed by OCT overnight. Samples were then snap frozen and 6 μm sections taken sectioned using a Leica 3050n cryostat. Sections were stained with Alcian blue, Toluidine blue and Safranin O using standard histological procedures.

2.8. MPC encapsulation in HA/PEG hydrogels

MPCs (P4-6) were encapsulated in gels containing 15 mg/ml HA$_{TYR}$, 16.5 mg/ml PEG$_{HPA}$, 0.25 U/ml horseradish peroxidase (HRP) and varying amounts of hydrogen peroxide (H_2O_2). Soluble PPS was added into the gels prior to crosslinking at a final concentration of 5 μg/ml with HA-PPS and HA-PPS$_{COOH}$ incorporated at concentrations of 16 and 9 μg/ml respectively to provide an equivalent concentration of PPS. For clarity all concentrations are denoted as the equivalent concentration of PPS. MPCs were resuspended to a concentration of 5×10^6 cells/ml in solution containing all of the components except H_2O_2 and thoroughly mixed. Crosslinking was then initiated by the addition of H_2O_2 and the gels spotted out into low-binding tissue culture plates. After 15 min, growth media was added to the well and the cell/gel composites cultured with media changes every 3 days.

2.9. Histological analysis of HA/PEG hydrogels

MPCs were cultured in hydrogels for 21 days after which the samples were rinsed in PBS and fixed with 4% paraformaldehyde. Samples were prepared for histological analysis by dehydrating through a graded series of ethanols followed by xylene. Samples were embedded in paraffin and 8 μm sections cut. Staining for Haematoxylin and Eosin, Alcian blue, Safranin O and Picrosirius red was performed on dewaxed sections according to standard protocols.

2.10. Immunostaining

Sections were rehydrated to water then incubated in 0.01% (w/v) pepsin at 37 °C followed by 0.1% (w/v) hyaluronidase for 1 h then 0.1% triton-X-100 for 5 min, both at room temperature. Samples were blocked with 2%BSA/2% goat serum in Tris-buffered saline (TBS) prior to incubation with primary antibodies (rabbit anti-Collagen-I, CL50IIIAP and mouse anti-Collagen-II, Ab3092). Staining was detected with Alexa fluor-conjugated secondary antibodies and samples co-stained with Hoechst 33342. Samples were mounted in Vetashield (Vector laboratories) and imaged using Olympus IX81 microscope.

3. Results

3.1. Synthesis and characterization of covalently linked HA and PPS

To facilitate the tethering of PPS into the HA/PEG hydrogel system, strategies to covalently link PPS to the HA$_{TYR}$ backbone of the hydrogels were developed. PPS was bound to HA$_{TYR}$ using EDC/NHS chemistry to link acid groups on PPS with amine groups on HA. In all cases HA, which had been previously functionalised with tyramine to facilitate crosslinking into hydrogels (HA$_{TYR}$), was further modified with cystamine hydrochloride to provide amine groups for further coupling to PPS. In one strategy, this coupling was mediated by the pendant acid group which is present every 10th repeat in the PPS polymer. However, an additional strategy using a form of PPS that had been modified by the addition of carboxyl groups (thereby providing additional acid groups for the coupling reaction), was also tested (Fig. 1). The products from these reactions were named HA-PPS and HA-PPS$_{COOH}$, respectively.

Carboxylation of PPS was performed using chloroacetic acid to add acid groups to PPS through formation of an ether bond between the chloroacetic acid and the sulphate groups on the PPS. The success of this reaction was confirmed by FTIR with the gain of a peak at 1720, corresponding to the C=O stretch from the acid group (Fig. 2A). The synthesis of amine-functionalised-HA$_{TYR}$ (HA$_{tyr}$-NH$_2$) was also confirmed by FTIR, whereby a peak at 1700–1750, demonstrating the formation of an amide bond linking the HA$_{TYR}$ and cystamine groups, was observed in the HA$_{tyr}$-NH$_2$ spectra (Fig. 2B).

Further analysis using a TNBSA assay to detect amine groups, showed an increase in amine groups after amine functionalisation of HA$_{TYR}$ (Fig. 3A). Quantitation showed a degree of functionalisation of 8% of the available acid groups in the HA molecule, providing
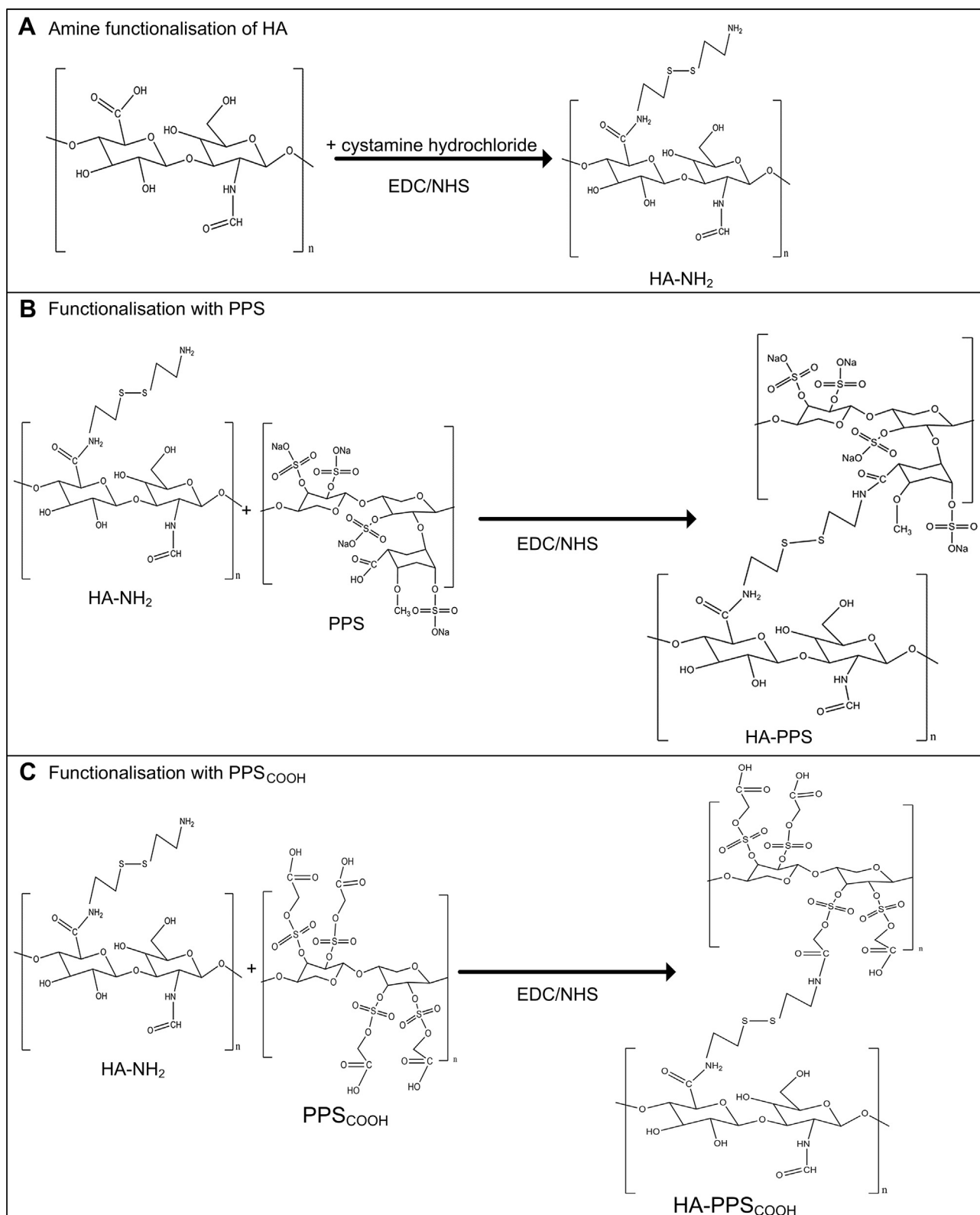
Fig. 1. Reaction scheme to show strategies for coupling HA and PPS. Covalent coupling of HA and PPS was achieved using EDC/NHS to couple amine groups on (A) amine-functionalised HA to acid groups on (B) PPS or (C) carboxylated PPS (PPS$_{COOH}$).

a large number of groups available to bind the PPS. Subsequent testing of HA$_{tyr}$–NH$_2$ after coupling with PPS or PPS$_{COOH}$ indicated a decrease in the number of amine groups to less than 1%, indicating successful coupling to PPS. 1H NMR analysis of HA-PPS and

HA-PPS$_{COOH}$ was also used to confirm the direct coupling of PPS to HA. This showed a degree of substitution of 3% for HA-PPS and 8% for HA-PPS$_{COOH}$. Gradient NMR indicated that approximately three-quarters and two-thirds of this was directly bound in the HA-PPS
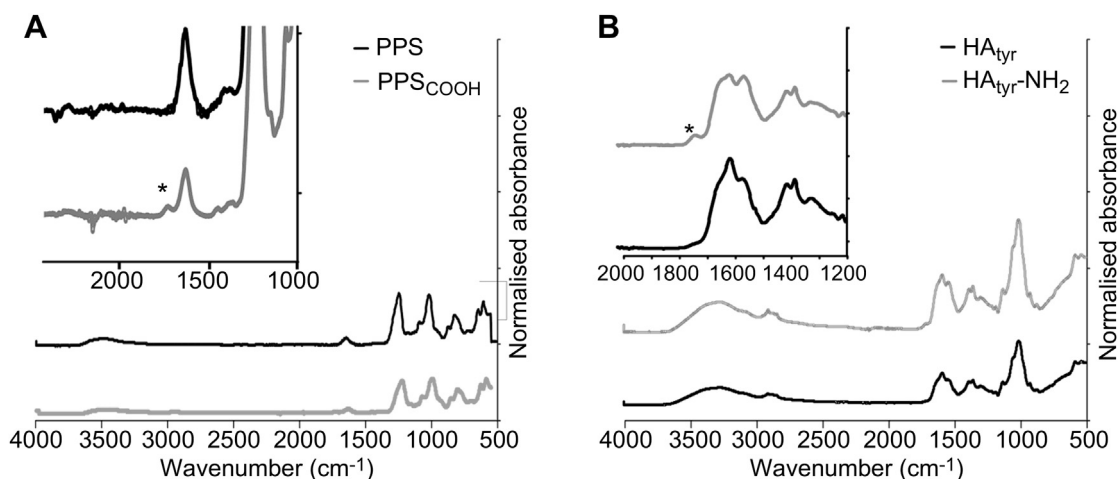
Fig. 2. FTIR analysis of carboxylated PPS and amine-functionalised HA. (A) ATR-FTIR overlays of PPS (black) and PPS$_{COOH}$ (grey). The inset shows the region from 1000 to 2500 with the C=O stretch peak at 1720 denoted by a star. (B) ATR-FTIR overlays of HA$_{TYR}$ (black) and HA$_{TYR}$-NH$_2$ (grey). The inset shows the region from 1200 to 2000 with the peak at 1700–1750 (black star) corresponding to the formation of an amide bond.

and HA-PPS$_{COOH}$ respectively (Fig. 3B and C). These reactions therefore produced two different molecules in which PPS was covalently bound to HA$_{TYR}$, providing a means to directly tether PPS into our hydrogel system.

3.2. Effects of bound vs. soluble PPS on MPC viability and proliferation

The HA-PPS and HA-PPS$_{COOH}$ conjugates were compared to unbound (soluble) PPS for their effects on MPC proliferation as assessed using a CCK8 assay. After 5 days culture, significantly fewer cells were detected in samples treated with PPS, HA-PPS and HA-PPS$_{COOH}$ compared with untreated controls. This effect was dose-dependent, in a manner inversely proportional to the PPS concentration and the magnitude of the response was greater for cells treated with HA-PPS than either PPS or HA-PPS$_{COOH}$ (the number of MPCs treated with the highest concentration of HA-PPS was less than 50% of untreated controls). Samples treated with HA alone were not significantly different to untreated cells at any concentration (Fig. 4A).

To confirm that these changes were not caused by a decrease in cell viability, live/dead staining was performed on MPCs treated with PPS and HA-PPS for 5 days. This indicated high levels of cell viability (>99%) across all samples, confirming that there were no

detrimental effects from the PPS and HA-PPS treatments (Fig. 4B). Additionally, an EdU assay for cell proliferation showed a decrease MPC proliferation with increasing concentrations of PPS, HA-PPS and HA-PPS$_{COOH}$ (Table 1). Correlating very closely with the CCK8 results, the decrease in cell proliferation was most pronounced with HA-PPS treatment, followed by HA-PPS$_{COOH}$ and PPS. Again, no such effect was observed for MPCs treated with HA alone. Together these data suggest that PPS, HA-PPS and HA-PPS$_{COOH}$, do not impact upon MPC viability but reduce MPC proliferation.

Notably, when cultured in the presence of PPS, HA-PPS and HA-PPS$_{COOH}$, MPCs underwent changes in cell morphology, and displayed a more spread, polygonal shape than the classic fibroblastic morphology of untreated MPCs (Fig. 5A). These finding suggest that PPS, HA-PPS and HA-PPS$_{COOH}$, promote MPC differentiation rather than a proliferation. Consistent with initiation of chondrogenic differentiation, in these monolayer cultures expression of the chondrogenic factor BMP13 was elevated and the BMP-antagonist Noggin was decreased (Fig. 5B).

3.3. Effects of bound vs. soluble PPS on MPC differentiation

In a previous study [14], PPS was shown to enhance chondrogenic differentiation of MPCs. As PPS can induce chondrogenic differentiation of MPCs in the absence of the traditional media
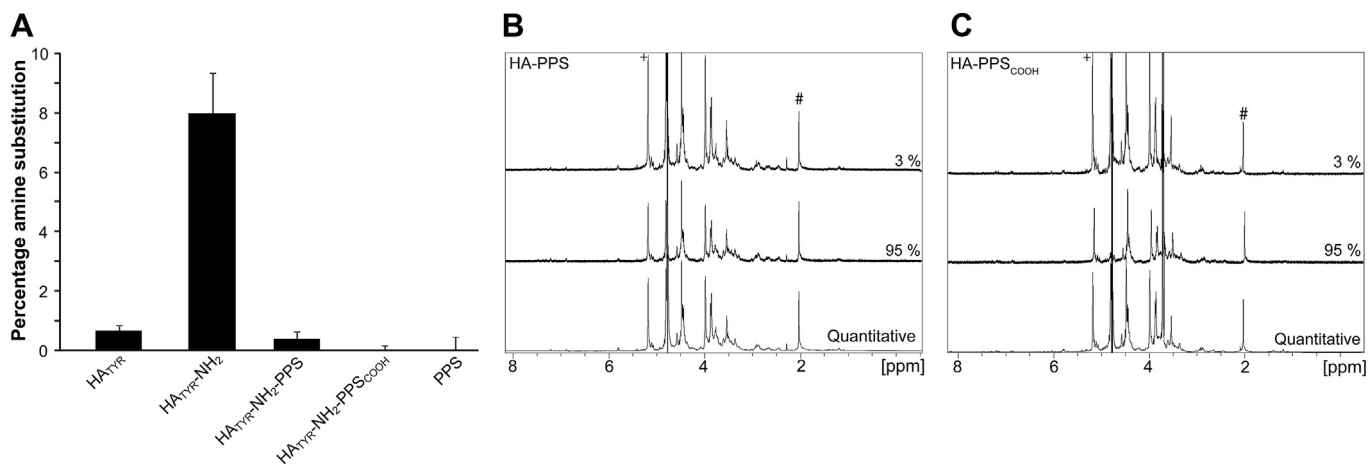
Fig. 3. Characterisation of HA-PPS coupling reactions. TNBSA assay showing the degree of substitution of free amine groups present on HA (A). 1H NMR spectra (1–8 ppm region) of (B) HA-PPS and (C) HA-PPS$_{COOH}$. The peaks for the H-1 anomeric proton of PPS (+) and the methyl protons of HA (#), which were used for quantitation are shown.
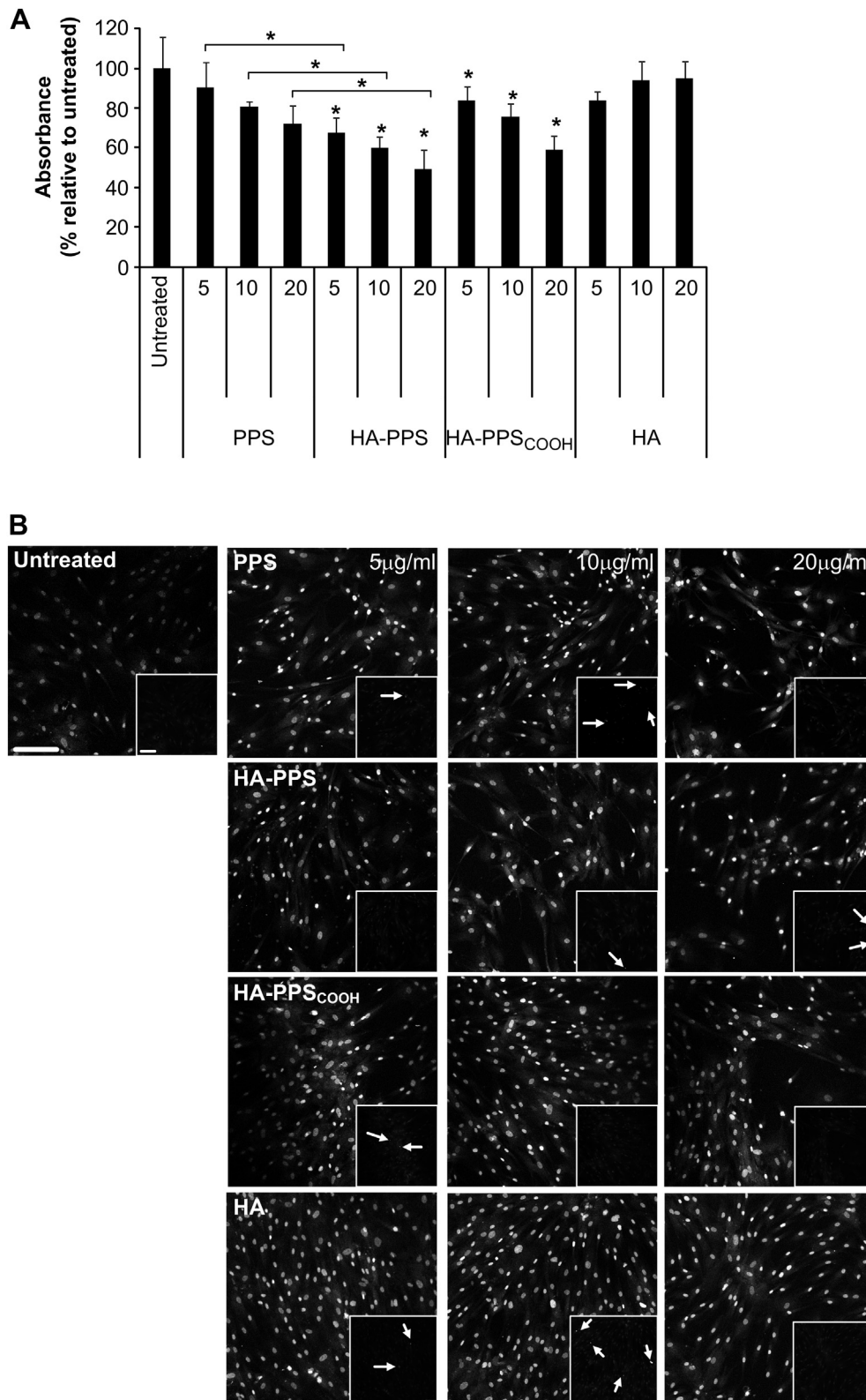
Fig. 4. Effect of PPS conjugates on MPC viability and proliferation. A) CCK8 assay of MPCs treated with PPS and HA-PPS for 5 days. Data is shown as mean ± SEM, $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) with the results for two independent MPC donors pooled together. B) Live/dead staining showing live cells (green) and dead cells (red). Scale bar = 50 um. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
EdU assay of MPC proliferation. The table shows the proportion of the MPC population that divided during a 24 h period pof treatment with EdU.

Treatment	Concentration	% EdU positive cells	
		Donor 1	Donor 2
Untreated	–	31.8	22.9
PPS	5 μg/ml	31.7	21.4
PPS	10 μg/ml	29.1	21.3
PPS	20 μg/ml	23.3	18.7
HA-PPS	5 μg/ml	23.4	20.5
HA-PPS	10 μg/ml	17.5	17.1
HA-PPS	20 μg/ml	12.5	15.2
HA-PPS$_{COOH}$	5 μg/ml	30	22.3
HA-PPS$_{COOH}$	10 μg/ml	24.9	21.5
HA-PPS$_{COOH}$	20 μg/ml	20.3	15.6
HA	5 μg/ml	28.3	24.2
HA	10 μg/ml	34.3	24
HA	20 μg/ml	33.4	21.5

supplements (including TGFβ), MPC pellets were cultured in MPC maintenance media supplemented with PPS, HA-PPS or HA-PPS$_{COOH}$, comparing the extent of differentiation to untreated controls or MPCs treated with HA alone.

A DMMB assay showed that GAG levels significantly increased when MPCs were treated with PPS or bound PPS, as compared to untreated cells or those treated with HA alone. Additionally, the effects of both HA-PPS conjugates were significantly greater than that of the unbound PPS. Unbound PPS increased GAG deposition to 150% of control levels whilst HA-PPS and HA-PPS$_{COOH}$ induced increases of 200% and 250% respectively (Fig. 6A).

Histological staining using Alcian blue and Safranin O was consistent with the DMMB assay, showing an increased intensity of staining for pellets treated with either unbound or bound PPS (Fig. 6B). Additionally, the pellets treated with bound PPS were the most compact and showed greater integrity of matrix formation than those treated with unbound PPS. Interestingly, while both the untreated pellets and those containing HA alone consisted of loosely aggregated matrix, those with HA were significantly smaller in comparison to any of the other conditions. Immunolabelling of type-I and type-II collagens showed an increase in collagen-II levels in all pellets treated with either PPS, bound PPS or HA. However, this was predominantly localized to the very periphery of the pellet for HA, whilst bound-PPS (especially HA-PPS) resulted in an even increase in collagen-II throughout the cell pellet. Pellets treated with PPS, HA-PPS$_{COOH}$ and HA also had high levels of collagen-I,
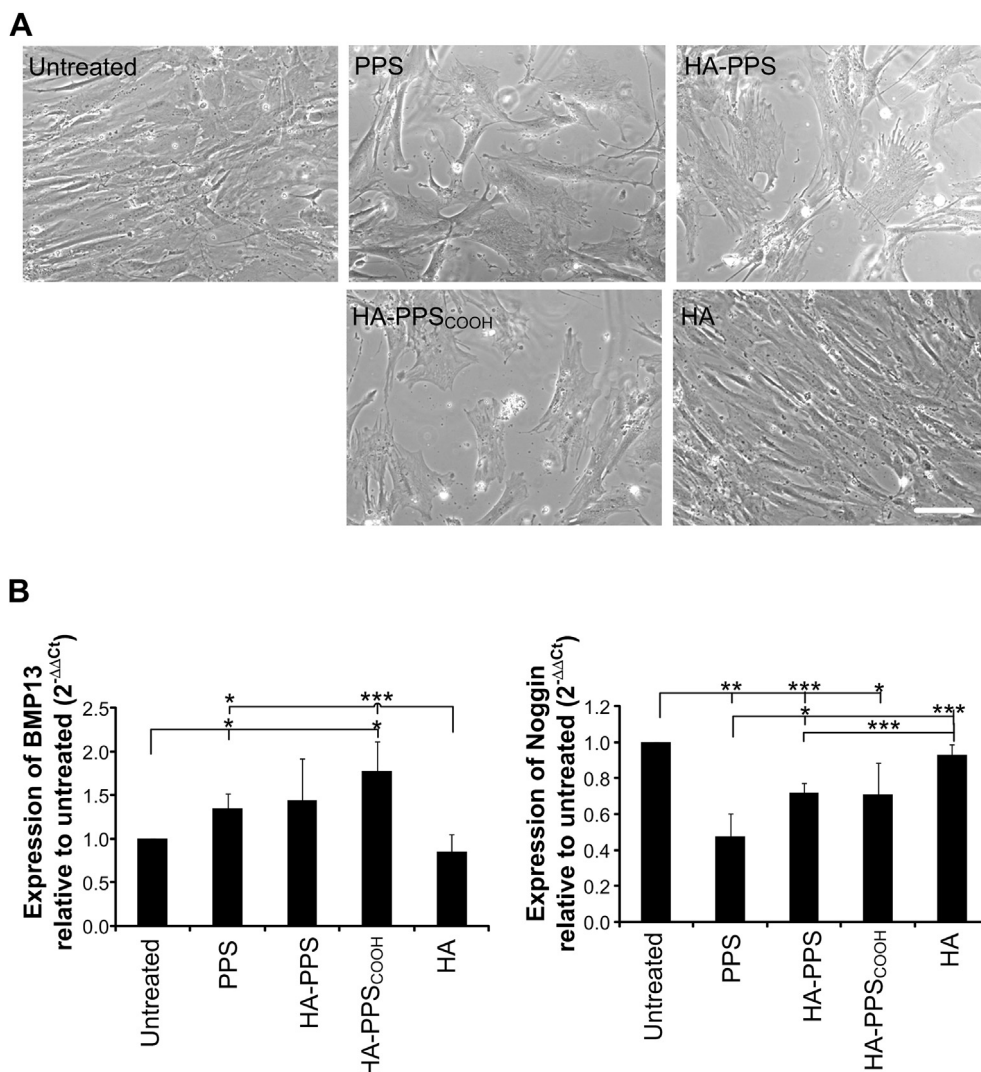
Fig. 5. Morphology and gene expression in monolayer MPCs treated with PPS conjugates. A) Phase contrast images of MPCs after 5 days treatment with PPS conjugates. Scale bar = 50 μm. B) qPCR determination of gene expression in MPCs treated with PPS and HA-PPS for 7 days. Pooled results from two independent donors are shown as mean (relative to DNA content) ± SD, $n = 8$ (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$.
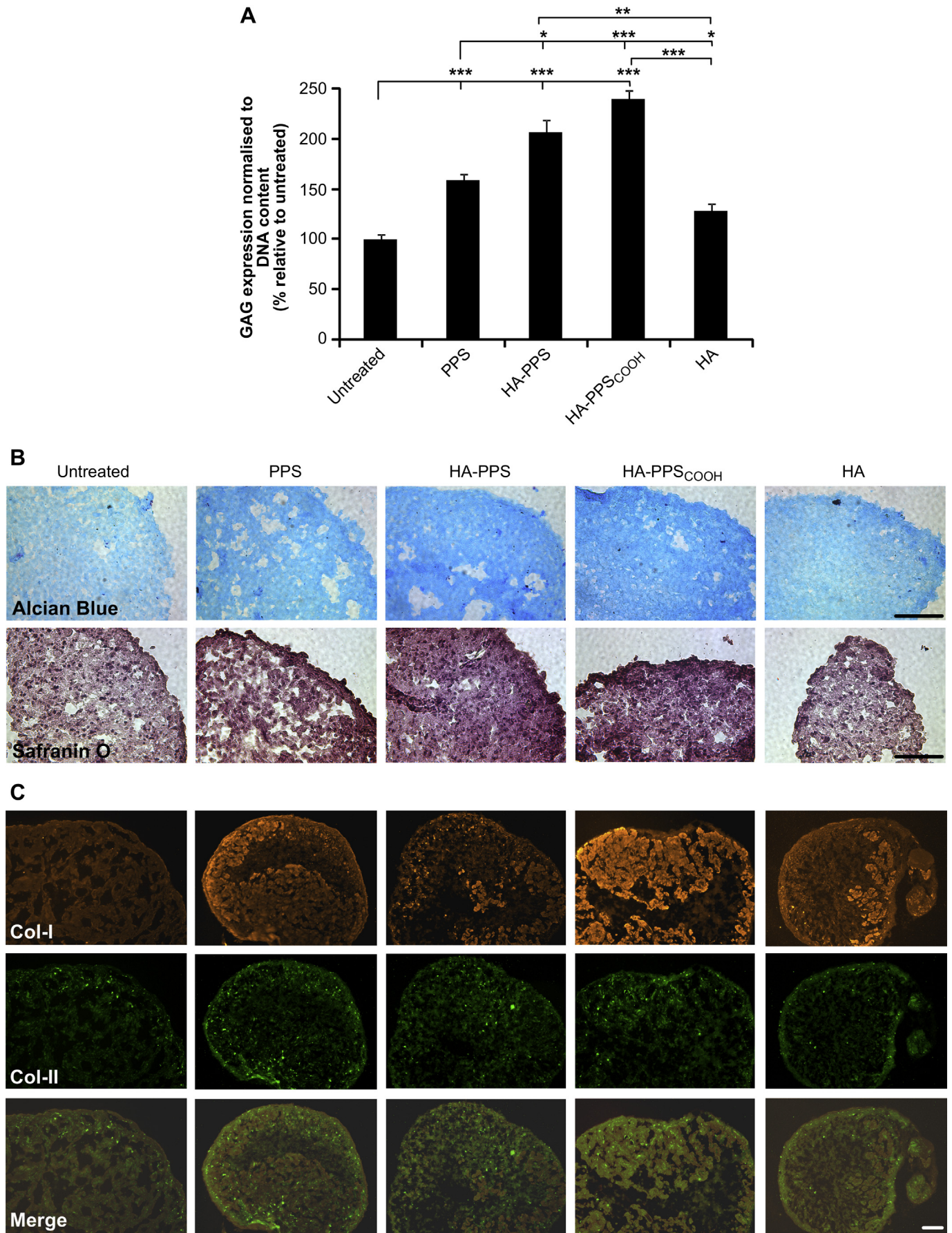
Fig. 6. Chondrogenic differentiation of MPCs treated with bound PPS conjugates. A) DMMB assay of GAG accumulation. Pooled results from two independent donors are shown as mean (relative to DNA content) $\pm$ SD, $n = 6$ (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$. B) Histological staining of day 21 micromass pellets. Scale bar $= 200$ μm. C) Immunolabelling of type-I and type-II-collagen after 21 days. Scale bar $= 200$ μm.

which was not present in HA-PPS pellets. This resulted in the HA-PPS pellets forming the most cartilage-like matrix, with large amounts of collagen deposition, but primarily type-II-collagen (Fig. 6C).

3.4. Characterisation of hydrogels incorporating soluble and bound PPS

Prior to encapsulating MPCs within hydrogels in which the HA-PPS was covalently linked to the matrix, the rheological properties of these gels were measured to determine whether the incorporation of the HA-PPS would affect the hydrogel mechanical properties. Comparisons were made between HA/PEG gels alone and those incorporating either 5 μg/ml soluble PPS or an equivalent level of HA-PPS (16 μg/ml HA-PPS contains the equivalent amount of PPS as 5 μg/ml soluble PPS). HA-PPS$_{COOH}$ was not included as there were no consistent differences in the response of MPCs to either HA-PPS or HA-PPS$_{COOH}$.

Time sweep analyses showed that the crosslinking kinetics were similar for all samples, whereby all of the gels reached an elastic shear modulus of 1 kPa within 6.5–8 min. However, the addition of bound PPS caused a decrease in the elastic modulus of the gels; the G′ at 5000 s decreased from ~5.5 kPa for both HA/PEG gels alone and those with soluble PPS, to 4.6 kPa for those incorporating HA-PPS. Frequency sweeps showed that the hydrogels were predominantly elastic materials (Fig. 7A) whilst stress sweeps (performed within the linear viscoelastic region) showed that higher levels of strain (10% compared to ~4%) could be applied to the gels incorporating bound PPS before failure of the material, as compared to the other gel formulations (Fig. 7B). The rheological properties of the hydrogels are summarized in Table 2.

The swelling and degradation profiles of the HA/PEG hydrogels were also determined and showed a high degree of swelling (over 20%) in all cases. However, the incorporation of both bound and soluble PPS into the HA/PEG hydrogels caused a significant decrease in the degree of swelling as compared to HA/PEG gels alone (Fig. 8A). There were no statistically significant differences between gels incorporating either the bound or soluble forms of PPS. Degradation profiles were also obtained over a 3 months period. These showed that, in the absence of cells, the hydrogel composition was stable with no significant degradation for any of the hydrogel compositions and no significant differences in degradation between the different gel compositions (Fig. 8B). It is important to note that this was performed in the absence of MPCs and therefore it would be expected that the breakdown of the hydrogels would be accelerated in the presence of cells due to the production of enzymes such as hyaluronidase. In support of this theory, some evidence of degradation was observed in gels when MPCs were encapsulated (data not shown) although at 3 weeks this was not enough to disrupt the overall composition of the cell/gel composites.

3.5. Cellular impacts of bound vs. soluble PPS in HA/PEG hydrogels

MPCs were encapsulated in the HA/PEG hydrogels and the viability and chondrogenic properties of the composites investigated. Live/dead staining of MPCs just 24 h after encapsulation confirmed that the crosslinking process did not affect MPC viability. Furthermore, very few dead cells were observed after 7 days indicating the longer-term viability of the MPC population when embedded within the hydrogels (Fig. 9A). There were no significant differences between any of the hydrogel compositions tested.

Histological analysis was used to examine the structure of the composites in which MPCs were encapsulated within the different HA/PEG hydrogels and to look for any evidence of ECM deposition relevant to NP tissue (Fig. 9B). H + E staining showed a similar overall structure for all of the samples, with MPCs distributed evenly throughout the hydrogels. Furthermore, after the 21 day
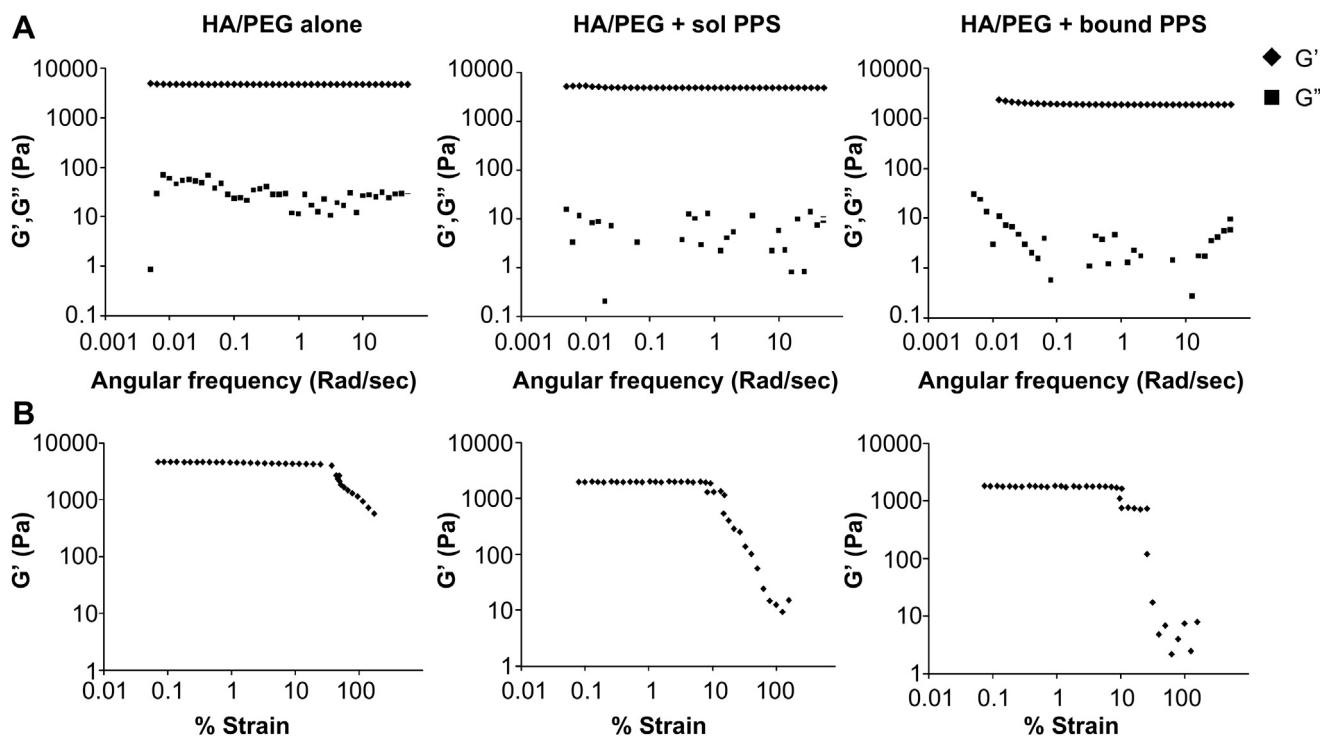

Fig. 7. Rheological characterization of HA/PEG hydrogels incorporating bound and soluble PPS. A) Frequency sweeps performed between 5.000E-3 and 50 rad/s at 1% strain. B) Oscillatory Stress Sweeps performed between 1 and 5 kPa at 50 rad/s. Reported as strain % on x-axis to highlight critical strain.

Table 2
Rheological properties of the HA-PEG hydrogels incorporating bound and soluble PPS.

Hydrogel type	PPS (µg/ml)	HA-Pps (µg/ml)	G′ at 5000 s (Pa)	G″ at 5000 s (Pa)	Critical strain (%)	Time to 1 kPa (s)
HA/PEG alone	0	0	5554	18.0	4.4	475
HA/PEG + soluble PPS	5	0	5463	12.2	4.0	501
HA/PEG + bound PPS	0	16	4641	23.9	10.1	399

culture period the MPCs seemed to have degraded the HA/PEG immediately surrounding the cell, to form a structure similar to the lacunae in which chondrocytes normally reside in cartilage and NP tissue. These were again highlighted by the Safranin O staining, in which a loss of red staining around the cells was suggestive of turnover of the pericellular matrix. Alcian blue staining was evident throughout all of the hydrogels. However, this was visibly more intense around the periphery of the MPCs encapsulated in hydrogels containing PPS, indicative of deposition of GAGs by the encapsulated cells. This was more prominent for the samples containing HA-PPS. Additional staining using Alcian blue at a lower pH (to provide greater specificity only for the most highly sulphated GAGs) again highlighted this (Fig. 9B, inset), with very little staining evident in blank gels and some staining in gels containing soluble PPS, but large areas of staining for those gels containing bound PPS. No significant differences were observed in Picrosirius red staining for any of the samples.

Immunostaining for type-I and type-II collagen showed deposition of both of these proteins by MPCs encapsulated in the HA/PEG hydrogels. With the addition of either soluble or bound PPS, there was a marked decrease in the level of Collagen-I and increase in Collagen-II, as compared to gels without PPS (Fig. 9C). The decrease in Collagen-I deposition was smaller for the bound PPS than soluble PPS but there was a greater amount of Collagen-II in these samples.

4. Discussion

The aim of the study, presented herein, was to covalently link PPS to HA and to compare the effects of the bound PPS and free PPS on encapsulated MPCs, when incorporated into HA/PEG hydrogels. We developed two reaction schemes for this purpose, using EDC/NHS chemistry to covalently link either PPS or carboxylated PPS (PPS$_{COOH}$) to amine-functionalised HA. Our data showed that both of these reaction schemes were successful in producing conjugates of HA and PPS. As expected, the efficiency of the reaction between HA-NH$_2$ and PPS$_{COOH}$ was higher than for unmodified PPS, given the increased number of acid groups available for the coupling

reaction when using PPS$_{COOH}$. The same strategies were also successful in coupling PPS to PEG$_{HPA}$ (data not shown), but this inhibited crosslinking of the HA/PEG hydrogels, most likely due steric hindrance from the large 5.7 kDa PPS molecule with the short arms of the 40 kDa 8-star-PEG$_{HPA}$.

Although PPS is similar in structure to a number of sulphated GAGs that exert their modulatory effects via the binding of growth factors (and has been shown to bind at least one growth factor, FGF2 [16]), the exact mechanism by which PPS influences MPC behaviour is currently unknown. In other cell types, there are indications that at least some of the effects of PPS may be mediated by receptor binding and internalization [26]. For this reason, it was essential to confirm that the activity of PPS was not abolished by linking it to the much larger HA polymer, which would likely inhibit internalization of the molecule and activation of these signalling mechanisms. Interestingly, although the results of proliferation and differentiation assays did show changes in PPS activity when conjugated to HA, this coupling did not simply reduce PPS function but altered its effects, in some cases making it more potent than unbound PPS.

No consistent differences in activity between the HA-PPS and the HA-PPS$_{COOH}$ were observed. This is important as the different coupling chemistries altered the PPS structure in different ways. Linking PPS to HA via the pendant acid group would have caused minimal changes to the PPS structure. However, the method used for PPS carboxylation added carboxyl groups at the expense of some of the sulphate groups that are likely to be important mediators of interactions of PPS with growth factors. For this reason, it is of note that binding via this method still resulted in a conjugate with biological activity, however this may be explained by the fact that not all of the sulphate groups would have been modified by the carboxylation reaction.

In general, both HA-PPS conjugates reduced MPC proliferation but promoted differentiation to a greater extent than unbound PPS. Although low concentrations of PPS are known to enhance MPC proliferation, this effect is concentration dependent and so the decrease we observed is in line with previous data using unbound PPS at concentrations of 5 µg/ml and above [14]. It is also consistent
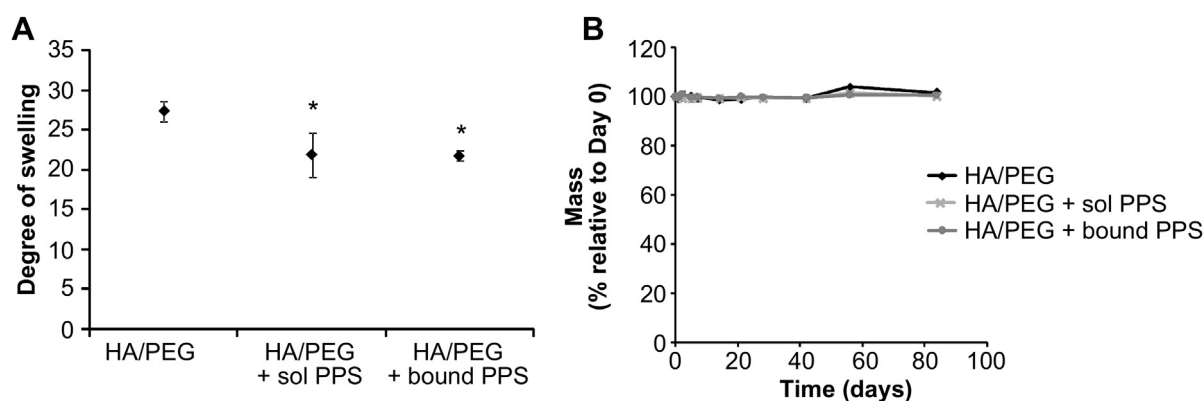
Fig. 8. Swelling and degradation properties of HA/PEG hydrogels with and without PPS. A. Degree of swelling of HA/PEG hydrogels. Data is presented as mean ± SD, n = 3. B. Degradation profiles of HA-PEG hydrogels.
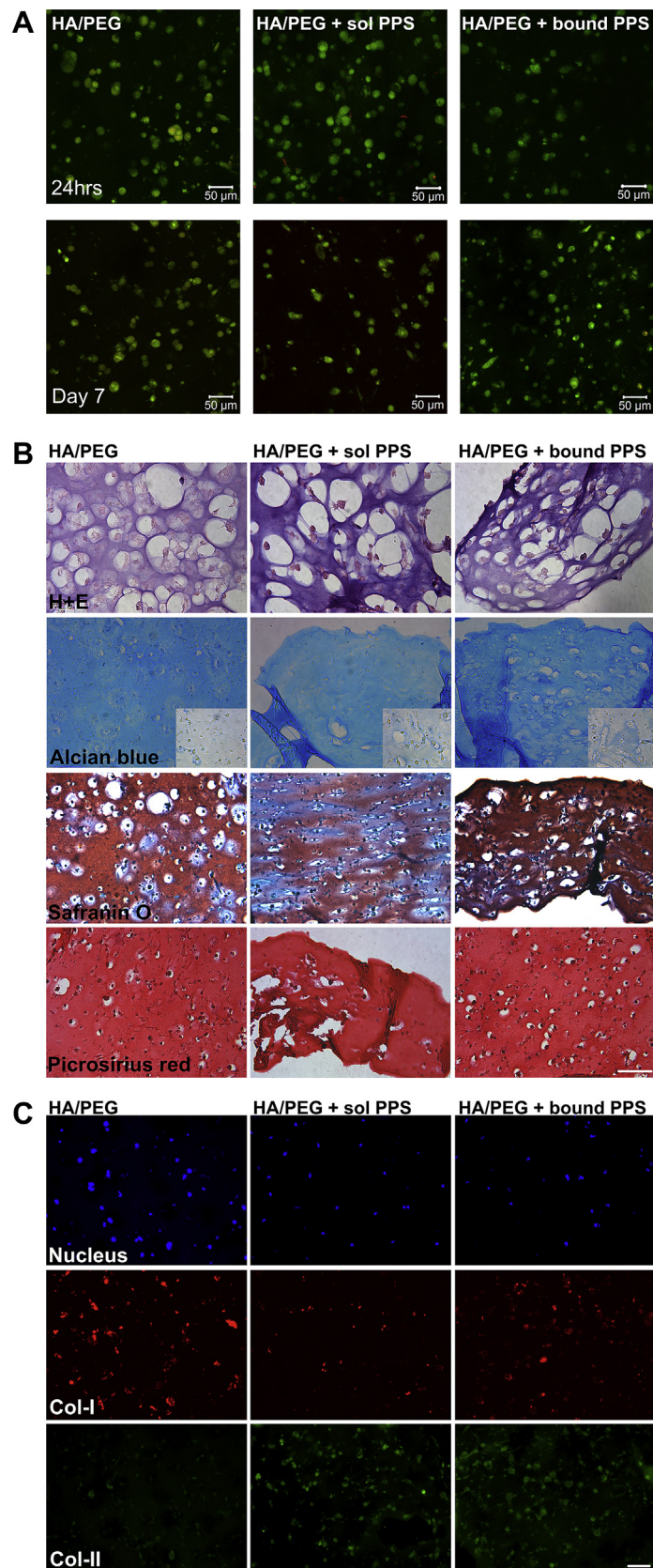
Fig. 9. Encapsulation of MPCs in HA/PEG hydrogels with and without PPS. A) Live/dead staining of encapsulated MPCs. Scale bar = 50 μm. B) Histological analysis of sections of hydrogels after 21 days culture. Scale bar = 50 μm. C) Immunostaining of hydrogel sections showing Collagen-I (red), Collagen-II (green) and Hoechst (blue). Scale bar = 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with data regarding the influence of unbound PPS on the proliferation of cell types other than MPCs [16]. As the rate of chondrogenesis was enhanced, our data suggest that, under these same concentrations, the bound PPS induced a switch from a proliferative to a differentiating phenotype. This correlates well with the changes we observed in cell morphology as well as the data from the chondrogenic assays.

Given the likely growth factor-binding role of both bound and soluble PPS, it may be that these differences in cellular response can be explained by differences in growth factor-binding affinity; however confirmation of this hypothesis requires further analysis. In support of this theory, GAG species other than PPS (for example heparin sulphate and chondroitin sulphate) have been shown to promote mesenchymal cell differentiation in a manner that has primarily been attributed to their ability to bind growth factors [9,16,19,24]. Irrespective of the mechanism of action, these results are of great interest as they suggest that the conjugation of PPS to HA may not only be useful for the presentation of PPS in our hydrogel matrix, but that the bound PPS may represent a novel additive to enhance MPC differentiation. Wider applications include the use of bound PPS as a culture supplement or a coating for other chondrogenic scaffolds where bound PPS has significant potential to replace more costly growth factors (such as TGFβ) in larger scale cultures or for future clinical applications.

Prior to the encapsulation of MPCs within hydrogels containing bound PPS, rheological characterization was performed to ensure that the incorporation of the bound PPS did not have a large effect on hydrogel mechanical properties. This was deemed important because mesenchymal stem/stromal cells are known to be sensitive to mechanical cues from their surrounding microenvironment [27,28]. The observed marginal decrease in hydrogel modulus in the presence of both soluble and bound PPS was likely a result of steric hindrance preventing the formation of some crosslinks. However, with the magnitude of the change at less than 1 kPa, this was not large enough to explain any differences in cellular response. Furthermore, the gels incorporating both bound and soluble PPS were able to withstand a much greater degree of strain before fracture than the HA/PEG gels alone. This may be a result of ionic interactions of the PPS or HA-PPS across networked chains which would enhance the toughness of the gels. Swelling and degradation studies also confirmed that the incorporation of bound PPS did not have any detrimental effects on hydrogel properties, thereby confirming their applicability for comparison to blank HA/PEG gels or those incorporating unbound PPS.

As suggested by previous work using HA/PEG hydrogels with unbound PPS, MPC viability was high when encapsulated in HA/PEG gels of all compositions. This is likely due to the rapid consumption of H_2O_2 by the crosslinking reaction, as no cell death was observed as a result of the crosslinking process. Additionally, cell viability was maintained with prolonged culture, which further demonstrates the utility of the system.

By covalently binding PPS to HA prior to incorporating it into the hydrogel, we hoped to more closely mimic the presentation of GAGs *in vivo*, where they interact with, and are consequently immobilised by, other ECM molecules [29]. Despite the fact that cell viability was equivalent for all hydrogel compositions, the observed increase in chondrogenic matrix deposition with bound-PPS supports this hypothesis. Of particular note is the increase in Collagen-II and decrease in Collagen-I with PPS and particularly bound-PPS. These data are promising in that Collagen-II is a major constituent of the NP and any construct aiming to regenerate NP tissue should increase Col-II and decrease Col-I levels. To the best of our knowledge, this is the first time that such an approach has been taken for cartilage/nucleus pulposus regeneration. Hydrogels have also been used as delivery vehicles for a variety of factors, for example with

the covalent binding of Dexamethasone [30] or TGFβ [22] into PEG hydrogels to induce osteogenic differentiation of MSCs. Commonly, these covalently bound drugs are released from the hydrogel as the polymer network degrades by direct hydrolysis of the tethering group between the drug and the polymer backbone, and in a similar manner, it is possible that there is some release of PPS from our system as the hydrogel degrades. Other hydrogels have been developed using decellularised matrix to ensure the inclusion of GAG molecules [23,31]. However, this approach results in an undefined mixture of matrix components, which is undesirable for clinical applications. For this reason, a strategy such as the one described here has greater potential by allowing the incorporation of precise levels of specific components, which may in turn enhance our ability to *direct* the biological outcomes of encapsulated cells.

5. Conclusion

The results of this investigation confirm that we have successfully coupled PPS to HA to facilitate covalent presentation of PPS to MPCs encapsulated within HA/PEG hydrogels. We confirmed not only that the new conjugate molecule retained its bioactivity, but that in many cases it outperformed unbound PPS. When incorporated into hydrogels, the bound PPS showed enhanced potential for cartilage formation and so may offer a promising strategy for the regeneration of NP tissue. However, through its use as a coating for other scaffold types or as a media supplement, HA-PPS may also have much broader applications and offers significant promise for a wide range of tissue engineering applications.

Acknowledgements

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