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Enzymatic co-crosslinking of star-shaped poly(ethylene glycol) tyramine and hyaluronic acid tyramine conjugates provides elastic biocompatible and biodegradable hydrogels

Rong Wang [1], Xiaobin Huang [2], Bram Zoetebier, Pieter J. Dijkstra, Marcel Karperien [*]

Department of Developmental BioEngineering, Faculty of Science and Technology, Tech Med Centre, University of Twente, P.O. Box 217, 7500 AE, Enschede, the Netherlands

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ABSTRACT

A combination of the viscoelastic properties of hyaluronic acid (HA) and the elastic properties of star shaped 8-arm poly(ethylene glycol) (8-arm PEG) was used to design in-situ forming hydrogels. Hydrogels were prepared by the enzymatic crosslinking of a partially tyramine modified 8-arm PEG and a tyramine conjugated HA using horseradish peroxidase in the presence of hydrogen peroxide. Hydrogels of the homopolymer conjugates and mixtures thereof were rapidly formed within seconds under physiological conditions at low polymer and enzyme concentrations. Elastic hydrogels with high gel content (≥95%) and high storage moduli (up to 22.4 kPa) were obtained. An in vitro study in the presence of hyaluronidase (100 U/mL) revealed that with increasing PEG content the degradation time of the hybrid hydrogels increased up to several weeks, whereas hydrogels composed of only hyaluronic acid degraded within 2 weeks. Human mesenchymal stem cells (hMSCs) incorporated in the hybrid hydrogels remained viable as shown by a PrestoBlue and a live-dead assay, confirming the biocompatibility of the constructs. The production of an extracellular matrix by re-differentiation of encapsulated human chondrocytes was followed over a period of 28 days. Gene expression indicated that these highly elastic hydrogels induced an enhanced production of collagen type II. At low PEG-TA/HA-TA ratios a higher expression of SOX 9 and ACAN was observed. These results indicate that by modulating the ratio of PEG/HA, injectable hydrogels can be prepared applicable as scaffolds for tissue regeneration applications.

1. Introduction

In-situ forming hydrogels prepared by the enzymatic crosslinking of polymer conjugates received increasing attention for tissue regeneration applications during the past decade. Especially, the enzyme horseradish peroxidase (HRP), which has a high substrate specificity for phenolic residues present in tyramine and tyrosine, was extensively studied. Up to now the crosslinking of polymer conjugates was mainly directed to natural polysaccharides like e.g., dextran, hyaluronic acid, heparin and chitosan [1–16]. The fast covalent coupling of tyramine residues through radical generation by the enzyme in the presence of hydrogen peroxide made in-situ hydrogel formation possible within seconds and these systems are nowadays also named injectable hydrogels. Although concerns on the use of this plant enzyme and hydrogen peroxide have been posed, the very low concentrations of the catalyst and reagent necessary revealed no cytotoxic effects on cells in contact with the hydrogels or when cells were embedded into the hydrogel constructs [17,18].

The versatility of the system can be illustrated by more recent studies, which are on the basis of the work of Kaplan and co-workers [19] who showed that poly(ϒ-glutamine acid) conjugated with tyramine or tyrosine were rapidly crosslinked into hydrogels by HRP/H_2O_2.

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* Corresponding author.
E-mail address: marcel.karperien@utwente.nl (M. Karperien).
[1] Present address: Radboudumc, Dept. of Dentistry-Regenerative Biomaterials, Radboud University Medical Center, Philips van Leydenlaan 25, 6525 EX Nijmegen, The Netherlands.
[2] Present address: Department of Oral and Maxillofacial Surgery, School of Dental Medicine, University of Pennsylvania, 240 South 40th Street, Philadelphia, PA 19104-6030, US.

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Based on these results tyramine has become the main choice for end-group modification of synthetic water-soluble polymers or grafted to natural polymers. Concerning the latter, next to dextran, hyaluronic acid and chitosan tyramine conjugates, alginate, celluloses, and proteins like gelatin and albumin were studied for their application as injectable hydrogels [20–29]. Natural polymers containing carboxylic acid groups can be conjugated with tyramine after activation methods. In case of polymers containing free amine groups, like in chitosan or gelatin, conjugation is performed with an activated hydroxyphenyl alkanoic acid.

Synthetic water-soluble polymers conjugated or end-functionalized with tyramine groups received minor attention up to now. Sakai and co-workers studied hydrogels based on tyramine conjugates of poly (vinyl alcohol) as wound dressing materials [30]. They developed a method for the in-situ generation of hydrogen peroxide by the enzymatic oxidation reaction of glucose in the present of glucose oxidase. Controlling the concentration of the hydrogen peroxide during the cross-linking process is beneficial because HRP is inactivated at higher concentrations of H_2O_2. Park and co-workers described the use of tyramine coupled to end-functionalized four arm poly(propylene oxide)-poly(ethylene oxide) copolymers. These materials could be degraded via hydrolysis of labile esters linking units next to the PEO chain. Linear mono tyramine end-functionalized PEGs have been grafted to gelatin and hydrogels were prepared by co-crosslinking with hyaluronic acid. Importantly, it was also shown in these studies that peptides containing tyrosine residues can be co-crosslinked in the networks providing bioactivity to the hydrogels [26–28,31,32].

The highly efficient and rapid enzymatic crosslinking reaction of polymer tyramine conjugates triggered researchers in studies towards the supramolecular engineering of hydrogels. Inclusion complexes of cyclodextrin and PEG were co-crosslinked with chitosan providing materials with controlled degradation profiles [33]. In a paper by the group of Haag and co-workers the temperature dependency on the kinetics of the crosslinking reaction of 3-(4-hydroxyphenyl)propionic acid (HPA) functionalized hyperbranced polyglycerol (hPG) was shown. They showed that the enzyme concentration and temperature could be used as tools to control the reaction kinetics and thereby the ultimate mechanical properties of the hydrogel [34].

Hyaluronic acid is an important component of the extracellular matrix in many tissues and is an attractive material for a variety of biomedical applications. HA plays a central role in protein adhesion and provides attachment sites for e.g., chondrocytes. Hydrogels comprising HA generally show viscoelastic properties, good water retention, and interaction with cells [35–37]. Synthetic hydrophilic polymers like PEGs with controlled molecular weight and end-groups are nowadays commercially available. Among the large number of PEGs nowadays available, the star shaped 8-arm PEG has shown a versatile hydrophilic component in a variety of physically and chemically crosslinked hydrogels and is generally elastic. Combining the viscoelastic properties and cell interactions of hyaluronic acid and the generally elastic properties of the star shaped 8-arm poly(ethylene glycol) (8-arm PEG) was regarded favorable as in-situ forming hydrogels in tissue regeneration applications. These properties might create a suitable compatible environment for cells. In previous research it was shown that HA can be conjugated with tyramine groups. The 8-arm PEG containing hydroxyl end-groups was selected for its long-term in vivo inertness and possible tyramine coupling after activation with a chloroformate. The preparation of enzymatically crosslinked hydrogels based on these PEG-tyramine and HA-tyramine conjugates was investigated in the presence of HRP and H_2O_2. The properties of the hydrogels, such as gelation time, gel content, swelling/degradation and mechanical properties were studied. Moreover, human mesenchymal stem cells were incorporated in these hydrogels to evaluate their cytocompatibility and human chondrocytes were incorporated to evaluate their proliferation and differentiation.

2. Materials and methods

2.1. Materials

The 8-arm poly(ethylene glycol) (8-arm PEG, hexaglycerol core, Mw = 20,000) was acquired from Jenkem Technology (Allen, Texas, USA) and purified before use by dissolution in dichloromethane and precipitation in cold diethyl ether. Hyaluronic acid sodium salt (Mw = 15–25 kg/mol) was purchased from Contipro. The polymer was dried by lyophilization overnight before use. *p*-Nitrophenyl chloroformate (PNC, 96%) was purchased from Sigma-Aldrich (Zwijndrecht, the Netherlands) and was sublimated before use. Anhydrous dimethylformamide (DMF, 99.8%), tyramine (99%), anhydrous pyridine (99.8%), hydrogen peroxide (H_2O_2, 30 wt% in H_2O), lithium chloride and HRP (261 U/mg) were obtained from Sigma-Aldrich and used without further purification. Dichloromethane (stabilized by Amylene, ≥99.9%), ethanol (99.8%) and diethyl ether (stabilized by BHT, ≥99.5%) were obtained from Biosolve (Valkenswaard, The Netherlands) and used as received. Dialysis membranes (Spectra/Por molecular porous membrane tubing 6, MWCO: 1 kDa) were acquired from Spectrum Laboratories, Inc. (Breda, The Netherlands) and rinsed with distilled water before use. Deuterated solvents were purchased from Aldrich. Phosphate buffered saline (PBS, 150 mM, pH 7.4) was purchased from Gibco Life Technologies Ltd (Paisley, UK).

2.2. Synthesis

PEG-OH_8 was firstly activated with p-nitrophenyl chloroformate (PNC) to form *p*-nitrophenyl carbonate conjugates (PEG-PNC_5-OH_3) as previously reported [38]. Hyaluronic acid-tyramine (HA-TA) was prepared as reported previously [8]. The method is added to the Supporting Information.

PEG-TA_5-OH_3. Typically, PEG-PNC_5-OH_3 (5.0 g, 1.2 mmol) was dissolved in 50 mL of anhydrous dichloromethane, and then tyramine (0.33 g, 2.4 mmol) pre-dissolved in 5 mL of anhydrous DMF was added at room temperature. The solution was stirred under a N_2 atmosphere for 2 h. The product was precipitated in cold diethyl ether, followed by washing with cold ethanol and diethyl ether and then dried under vacuum for one day to give PEG-TA_5-OH_3 (yield 92%). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 2.7 (t, $NHCH_2\mathit{CH_2}$), 3.3–3.4 (dd, $NH\mathit{CH_2}CH_2$), 3.63 (PEG protons), 4.19 (t, $C\mathit{H_2}OCO$), 5.00 (s, N*H*), 6.79–6.99 (d, aromatic protons).

2.3. Analysis

NMR spectroscopy: NMR spectra were recorded on a Bruker Ascend™ III 400 NMR (Nanobay) Spectrometer working at 400.13 MHz for ^{1}H. The chemical shifts were calibrated against residual solvent signals. Peak multiplicity was denoted as s (singlet), d (doublet), dd (double doublet) and t (triplet).

Hydrogel formation and gelation time. Enzymatically crosslinked hydrogels (0.2 mL) of PEG-TA_5-OH_3/HA-$TA_{2.2}$ (50/50) at a polymer concentration of 5, 10 or 20 wt% were prepared in vials at room temperature. The gelation time vs enzyme concentration was evaluated by changing HRP concentrations. In a typical procedure, to 0.16 mL of PEG-TA_5-OH_3/HA-$TA_{2.2}$ (50/50, 12.5 wt%) in PBS, a freshly prepared HRP solution (20 μL, 25 U/mL) and H_2O_2 solution (20 μL, 0.3 wt% stock solution) were added and the mixture was gently vortexed. The final polymer, HRP and H_2O_2 concentrations were 10 wt%, 2.5 U/mL and 10 mM, respectively. The gelation time was determined by using the vial tilting method. The experiment was performed in triplicate.

Gel content and water uptake: To determine the gel content, samples of PEG-TA_5-OH_3, HA-$TA_{2.2}$ or PEG-TA_5-OH_3/HA-$TA_{2.2}$ hydrogels (0.2 mL, 10 wt%) were prepared as described above. The gels were lyophilized and weighted (W_d), then extensively extracted with 5 mL of dichloromethane for a week to remove un-crosslinked polymer. The

solvent was replaced twice. The hydrogel was washed 3 times with ethanol, demi water, and dried under vacuum to a constant weight (W_c). The gel content was expressed as $W_c/W_d \times 100\%$.

The water uptake of PEG-TA_5-OH_3, HA-$TA_{2.2}$ or PEG-TA_5-OH_3/HA-$TA_{2.2}$ hydrogels (0.2 mL, 10 wt%) was determined as follows. After lyophilization, the dry hydrogels (W_d) were immersed in 5 mL of PBS at 37 °C for 3 d to reach swelling equilibrium. Swollen samples were removed from PBS and after removal of surface water, the samples were weighted (W_s). The water uptake of the hydrogels was calculated as follows: $(W_s\text{-}W_d)/W_d \times 100\%$.

Rheology: Oscillatory rheology experiments were performed on PEG-TA_5-OH_3, HA-$TA_{2.2}$ or PEG-TA_5-OH_3/HA-$TA_{2.2}$ hydrogels with a concentration of 10 wt%. Hydrogels were in-situ formed using a Mix-Pac™ double chamber syringe system with a Medmix Systems AG ML-2.0-16-LLM mixing chamber (Winterthur, Switzerland). In a typical experiment, a polymer solution (0.16 mL, 12.5 wt% in PBS) mixed with a freshly prepared solution of H_2O_2 (40 μL, 0.3 wt% stock solution in PBS) was placed in one of the syringe chambers. Another polymer solution (0.16 mL, 12.5 wt% in PBS) was mixed with a freshly prepared HRP solution (40 μL, 25 U/mL stock solution in PBS) and placed in the other chamber. After the sample was injected onto the bottom plate, the upper plate was immediately lowered to a measuring gap size of 0.3 mm followed by a time sweep for 10 min at 25 °C. A frequency ω of 1 Hz and a strain γ of 1% were applied to minimize the influence of deformation on the hydrogels. Then, a strain sweep was recorded at a constant frequency ($\omega = 1$ Hz; $\gamma = 0.1$–50%) and after 10 min equilibration, a frequency sweep was recorded at constant strain amplitude ($\gamma = 1\%$; $\omega =$ 0.1–100 Hz) at 25 °C.

Unconfined compression tests: The mechanical properties of PEG-TA_5-OH_3, HA-$TA_{2.2}$ and PEG-TA_5-OH_3/HA-$TA_{2.2}$ hydrogels were tested using a Zwick-Roell Z020 tester (Germany) with a load cell of 250 N. The compressive strength of the hydrogels was measured at a strain rate of 1 mm/s. Standard cylindrical hydrogel samples were prepared using a PDMS mold (5 mm in height and 9 mm in diameter). The specimens were placed between two load cells and compressed until break. The Young's modulus was calculated from the slope of the linear region of the stress-strain curves (10–20% strain). For each sample, a minimum of three measurements was taken and the average value was reported. The nominal stress and strain, σ and ε, were recorded as the applied load divided by the original cross-sectional area of the samples and the displacement divided by L_0.

Morphology of gels: To visualize the morphology of PEG-TA_5-OH_3, HA-$TA_{2.2}$ or PEG-TA_5-OH_3/HA-$TA_{2.2}$ hydrogels, the samples were frozen in liquid nitrogen and then dried for two days. After coating with gold, samples were analyzed with a Zeiss HR-SEM equipped with NORAN EDS and WDS with a maximum and minimum accelerating voltage of 30 kV and 0.2 kV, respectively, and a field-emission gun.

Swelling and degradation behavior: PEG-TA_5-OH_3, HA-$TA_{2.2}$ or PEG-TA_5-OH_3/HA-$TA_{2.2}$ hydrogels (0.2 mL, 10 wt%) were prepared as described above and accurately weighted (W_i). The samples were subsequently incubated in 2 mL of PBS containing 100 U/mL hyaluronidase at 37 °C. At regular time intervals, the enzyme solution was removed from the samples and the weight of the hydrogels was determined (W_{de}). Remaining gel (%) was calculated from W_{de}/W_i. After weighing, fresh enzyme solutions were added to the hydrogels. The experiments were performed in triplicate.

Human mesenchymal stem cells isolation: All polymers were sterilized by filtration of aqueous solutions through filters with a pore size of 0.22 μm before lyophilization and thereafter stored in sterile tubes before use. Human bone marrow derived mesenchymal stem cells (hMSCs) were isolated as previously reported [39] and cultured in MSC proliferation medium (α-MEM (Gibco) supplemented with 10% fetal bovine serum, 1% L-glutamin (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. Hydrogels containing hMSCs were prepared under sterile conditions by mixing a polymer/cell suspension together with HRP and H_2O_2 solutions. PEG-TA_5-OH_3/HA-$TA_{2.2}$ solutions at different weight ratios (100/0, 80/20, 50/50, 20/80 or 0/100) were prepared in proliferation medium and HRP and H_2O_2 stock solutions were made in PBS. Human mesenchymal stem cells (P3) were dispersed in the precursor solution of the hydrogels. The hydrogels were then formed as described in section *hydrogel formation and gelation time*. The final polymer concentration was 10 wt% and the cell seeding density in the gels was 5×10^6 cells/mL. After gelation, the hydrogels (100 μL each) were transferred to a culture plate and 2 mL of hMSCs proliferation medium was added. The samples were incubated at 37 °C in a humidified atmosphere containing 5% CO_2. The medium was replaced every 3 or 4 days.

Cell viability and proliferation. The metabolic activity and viability of hMSCs incorporated in the hydrogels were determined by a PrestoBlue assay and Live-dead assay according to the manufacturer's protocols [40]. At day 3, 7, 14, 21 and 28 the hydrogel constructs were rinsed with PBS and 30 μL of PrestoBlue reagent in 270 μL of hMSCs proliferation medium was added. Following an incubation time of 1 h at 37 °C, the PrestoBlue medium was transferred to a 96-well plate and the fluorescence of each well was measured using a microplate reader (Victor) with excitation at 560 ± 10 nm and emission at 590 ± 10 nm. Data are presented as average ± SD (n = 3). Data were also statistically analyzed by using GraphPad Prism software. After removal of PrestoBlue medium, the hydrogel constructs were rinsed with PBS twice for 10 min each and then stained with calcein AM/ethidium homodimer using the Live-dead assay Kit (Invitrogen) according to the manufacturers' instructions. Hydrogel/cell constructs were visualized using a fluorescence microscope (EVOS). Living cells fluoresce green and the nuclei of dead cells red.

Chondrogenic differentiation of human chondrocytes in hydrogels: Human primary chondrocytes (hPCs) were obtained from full thickness cartilage dissected from knee biopsies of a patient undergoing total knee replacement as published previously [41] and cultured in chondrocyte expansion medium (DMEM with 10% heat inactivated fetal bovine serum, 1% penicillin/streptomycin (Gibco), 0.5 mg/mL fungizone (Gibco), 0.01 M MEM non-essential amino acids (Gibco) and 0.04 mM L-proline) at 37 °C in a humidified atmosphere (5% CO_2). The use of human material was approved by a local medical ethical committee. Cylindrically shaped hydrogels with cells encapsulated were prepared using a home-made Teflon mold containing wells (100 μL/well, sterilized before use). PEG-TA_5-OH_3/HA-$TA_{2.2}$ hydrogel-cell constructs at different polymer weight ratios (100/0, 80/20, 50/50, 20/80 or 0/100) were prepared by first mixing gel precursor solutions with chondrocytes (P3). The polymer concentration was 12.5 wt%. To each Teflon well 80 μL of the mixture was added. Thereafter, a freshly prepared HRP solution (10 μL of a 25 U/mL stock solution) and H_2O_2 solution (10 μL, 0.3 wt%) were mixed thoroughly, transferred to the wells and the mixture was quickly and gently shaken to allow gelation (10 s). The final cell density was 5×10^6 cells/mL and the final concentration was 10 wt%. All gel samples were then transferred to a 48 well plate and cultured in 1.0 mL of chondrogenic medium with transforming growth factor β3 (TGF-β3, R&D System). Chondrogenic medium consisted of high-glucose DMEM (Invitrogen), which was supplemented with 0.1 μM dexamethasone, 1% penicillin/streptomycin, 100 μg/mL sodium pyruvate, 0.2 mM L-ascorbic acid, 50 mg/mL insulin-transferrin-selenite (ITS+1, Sigma) and 10 ng/mL TGF-β3. Medium was changed every 3 or 4 days.

RNA isolation and real-time quantitative polymerase chain reaction (qPCR): After culturing the hydrogel/cell constructs in chondrogenic medium for 21 days, the samples were collected, washed with PBS and homogenized with a plastic grinding rod. Then 1 mL of Trizol reagent (Invitrogen, Carlsbad, CA) was added. Total RNA was isolated from the lysate according to the manufacturer's protocol [42]. The concentration of RNA was measured using the Nanodrop 2000 (Thermo scientific, Wilmington, USA). Subsequently, the RNA (1 μg) was reverse transcribed into single strand cDNA using the iScript Kit (BioRad)

Table 1
Polymerase chain reaction primers.

Primer	Direction	Sequence
Human aggrecan	Forward	5′ AGGCAGCGTGATCCTTACC 3′
	Reverse	5′ GGCCTCTCCAGTCTCATTCTC 3′
Human collagen type IIa1	Forward	5′ CGTCCAGATGACCTTCCTACG 3′
	Reverse	5′ TGAGCAGGGCCTTCTTGAG 3′
Human GAPDH	Forward	5′ CGCTCTCTGCTCCTCCTGTT 3′
	Reverse	5′ CCATGGTGTCTGAGCGATGT 3′

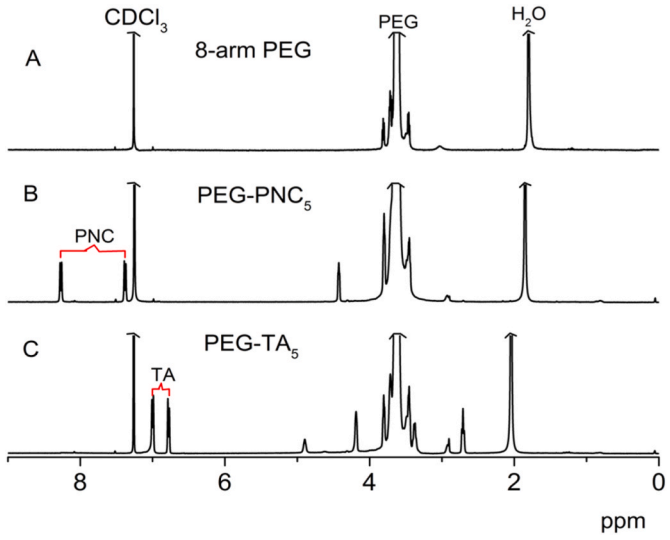

Fig. 1. ^{1}H NMR spectra (400 MHz, $CDCl_3$) of 8-arm PEG (A), PEG-PNC_5-OH_3 (B) and PEG-TA_5-OH_3 (C).

according to the manufacturer's recommendations. One microliter of each normalized cDNA sample was analyzed using the "SensiMix™ SYBR & Fluorescein kit" (Bioline) and a real-time PCR Cycler (Bio-Rad CFX96, Hercules, CA). The expression of collagen type II and aggrecan (Table 1) was analyzed and normalized to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For each reaction a melting curve was generated to test primer dimer formation and non-specific priming.

Histological analysis: Samples cultured for 28 days were fixed in 10% formalin for 2 h and then washed with PBS sucrose solution (20%) for 2 h at 4 °C. The samples were transferred to 1% PVA (Mw = 1 kDa) to reduce the formation of ice crystals when freezing for cryosectioning [43]. Constructs were then removed from the PVA solution, transferred to cryomolds with one flat plane facedown. Each cryomold was then filled with Cryomatrix™ (Thermo Scientific) and frozen in liquid nitrogen. After solidification, cryomolds were removed from the slurry and stored at −80 °C until cryosectioning. Routine cryosectioning was performed on the frozen blocks using a cryostat (Cryotome™ FE) at −20 °C. For each section, 10 μm cross sections along the flat plane of the construct were collected on SuperFrost® Plus Gold slides (Fisher Scientific). The slides were air-dried for several hours before Alcian Blue staining and Safranin-O staining.

Alcian blue staining: Proteoglycan deposition and distribution was visualized by staining sections with Alcian Blue. Typically slides were stained for sulfated glycosaminoglycans (GAG) with a 0.5 wt% solution of Alcian blue (pH = 1, adjusted with HCl) for 30 min. The samples were then counterstained with nuclear fast red (0.1 wt% of nuclear fast red in 5% aluminum sulfate) for 5 min to visualize nuclei. The slides were then washed with water and dehydrated.

Safranin-O staining: Safranin-O stains acidic proteoglycans present in cartilage tissues. Typically, slides were stained with a 0.1 wt% solution of Safranin-O for 5 min, and counterstained with haematoxylin for 4 min to visualize nuclei. The slides were then washed with water and dehydrated. Sections were analyzed using a bright field microscope (Hamamatsu Nanozoomer).

Statistical analysis. Statistical differences between two groups were analyzed using a Student's *t*-test. Those among three or more groups were analyzed using the One-way Analysis of Variance (ANOVA) with Tukey's post-hoc analysis. Statistical significance was set to a p value $\leq$ 0.01. Results are presented as mean ± standard deviation.

3. Results and discussion

3.1. Synthesis of PEG-TA_5-OH_3 and HA-$TA_{2.2}$

The synthesis of partially tyramine functionalized 8-arm poly (ethylene glycol) (PEG-$(OH)_8$) was performed by first reacting the hydroxyl groups of PEG-$(OH)_8$ with p-nitrophenyl chloroformate (PNC) to give PEG-PNC_5-OH_3 as previously reported [44]. Attempting to reach higher end-group activation using a large excess of p-nitrophenyl chloroformate resulted in increasing amounts of side products. Because the PEG-PNC_5-OH_3 could be obtained in high yield and purity no further attempts were made to increase the degree of the activation. Reaction of PEG-PNC_5-OH_3 with two times excess of tyramine (TA) afforded PEG-TA_5-OH_3 as determined from the relative integrals of TA (6.79–6.99 ppm) and PEG signals (3.63 ppm) (Fig. 1). The synthesis of tyramine conjugated hyaluronic acid (HA-$TA_{2.2}$) by EDAC/NHS activation of HA and reaction of the activated carboxylic acid groups with tyramine was previously reported [8]. In this one pot synthesis using an excess of activation reagents and tyramine, a degree of substitution of 2.2 was obtained, which means that approximately 5% of the carboxylic acid groups have been substituted with a tyramine group.

3.2. Hydrogel formation

Enzymatic crosslinking of aqueous solutions of the tyramine conjugated polymers PEG-TA_5-OH_3, HA-$TA_{2.2}$ and mixtures thereof using horseradish peroxidase in the presence of hydrogen peroxide readily afforded hydrogels. In previous research it was shown that at an HRP concentration of 15 U/mL and H_2O_2 concentration of 10 mM, hydrogels of dextran and hyaluronic acid were obtained within 1 min at concentrations ranging from 10 to 20 wt% [4]. The molar ratio of the H_2O_2 and tyramine as used in these experiments is 1:2, because every H_2O_2 molecule provides two phenolic radicals by the action of HRP. Higher concentrations of H_2O_2 may have a detrimental effect on the HRP whereas lower concentrations lead to incomplete crosslinking.

Applying similar concentrations of HRP, and a molar ratio of H_2O_2 to tyramine of 1:2 in initial gelation tests of PEG-TA_5-OH_3/HA-$TA_{2.2}$ (50/50) mixtures, instant gel formation was observed. In order to accomplish suitable gelation times for adequate mixing and easy handling the optimal concentration of HRP was determined in the 1–3 U/mL range at polymer concentrations of 5, 10 and 20 wt% (Fig. 2a). The gelation times of the polymer mixture PEG-TA_5-OH_3/HA-$TA_{2.2}$ 50/50 decreased from 40 s to 10 s with an increasing concentration of HRP from 1 to 3 U/mL. Interestingly, at the highest polymer concentration, somewhat longer gelation times were observed (15 s vs 10 s, $p < 0.05$), which may be due to the increased viscosity of solutions at higher polymer concentrations impairing mobility of the HRP. These initial experiments on the gelation kinetics of PEG-TA_5-OH_3/HA-$TA_{2.2}$ revealed fast gelation even at a very low polymer concentration of 5 wt% and more than 10 times lower HRP concentration compared to Dex-TA/HA-TA gels as previously reported.

The effect of the HRP concentration on the rheological properties of these PEG-TA_5-OH_3/HA-$TA_{2.2}$ 50/50 hydrogels were studied to set an optimal polymer concentration. Using a double syringe, hydrogels were in-situ formed at the plate of the rheometer and after lowering the measuring plate the storage and loss modulus were recorded. In Fig. 2b it is shown that at similar HRP concentrations, the storage modulus

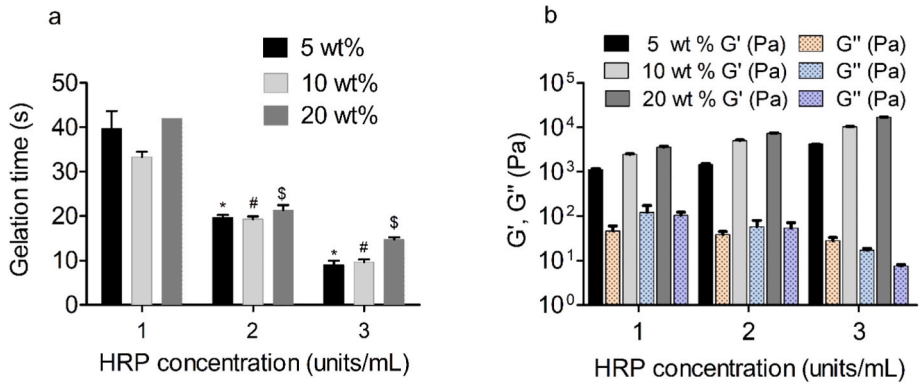

Fig. 2. a: Gelation time of 5–20 wt% PEG-TA_5-OH_3/HA-$TA_{2.2}$ (50/50) solutions as a function of HRP concentration. The H_2O_2 concentration was 10 mM. (n = 3, *$p < 0.05$ vs 5 wt%, 1–3 U/mL; #$p < 0.05$ vs 10 wt%, 1–3 U/mL; $ $p < 0.05$ vs 20 wt%, 1–3 U/mL.) b: the storage modulus (G′) and loss modulus (G″) of 5–20 wt% PEG-TA_5-OH_3/HA-$TA_{2.2}$ (50/50) solutions as a function of HRP concentration.

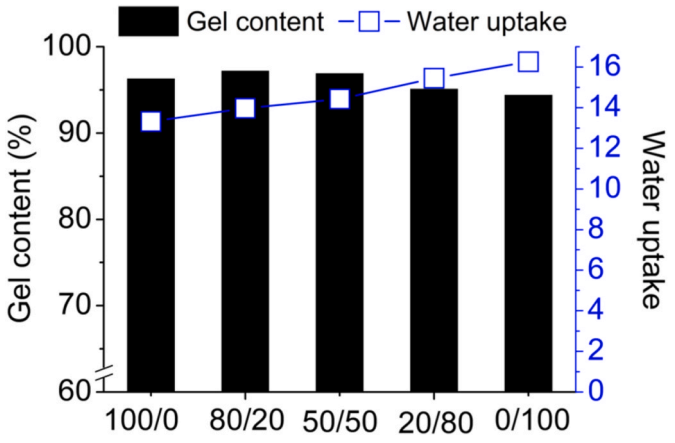

Fig. 3. Gel content and water uptake of 10 wt% PEG-TA_5-OH_3/HA-$TA_{2.2}$ hydrogels with various polymer compositions (100/0 to 0/100) at 10 wt%. Concentration of HRP was 2.5 U/mL and of H_2O_2 10 mM (molar ratio H_2O_2:TA was 1:2).

increased with increasing polymer concentration. At the same polymer concentration, the damping factor (G″/G′) decreased with increasing HRP concentration. Based on these experiments, the combination of a polymer concentration of 10 wt% and a HRP concentration of 2.5 U/mL were selected for all following experiments to allow adequate mixing and easy handling.

3.3. Hydrogel properties

The gel content and water uptake of PEG-TA_5-OH_3/HA-$TA_{2.2}$ hydrogels with ratios ranging from 100/0 to 0/100 were subsequently determined. The variation in gel content and water uptake of 10 wt% hydrogels is presented in Fig. 3. It was found that the gel content at all compositions was high (≥95%), indicating that almost all polymer chains were incorporated into the networks. The water uptake increased from 13.3 to 16.3 with increasing HA-$TA_{2.2}$ weight ratios which can be attributed to the polyanionic character of the hyaluronic acid.

The kinetics of hydrogel formation was followed by monitoring the storage modulus (G′) and loss modulus (G″) in time and results are presented in Fig. 4A. At all polymer ratios (PEG-TA_5-OH_3/HA-$TA_{2.2}$) no gelation point (defined as the crossover point of G′ and G″) could be

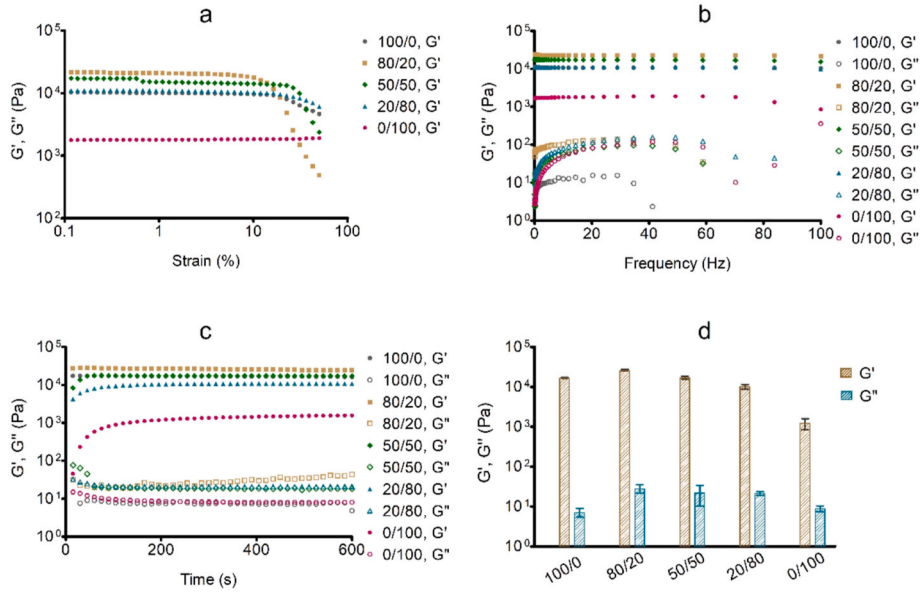

Fig. 4. a: Strain sweep of 10 wt % PEG-TA_5-OH_3/HA-$TA_{2.2}$ hydrogels; b: Frequency sweep of 10 wt % PEG-TA_5-OH_3/HA-$TA_{2.2}$ hydrogels; c: Storage moduli (G′) and loss moduli (G″) of 10 wt % PEG-TA_5-OH_3/HA-$TA_{2.2}$ hydrogels as a function of time; d: Corresponding storage moduli (G′) and loss moduli (G″). Hydrogels were prepared at a H_2O_2 concentration of 10 mM and a HRP concentration of 2.5 U/mL. All measurements were performed at 25 °C.

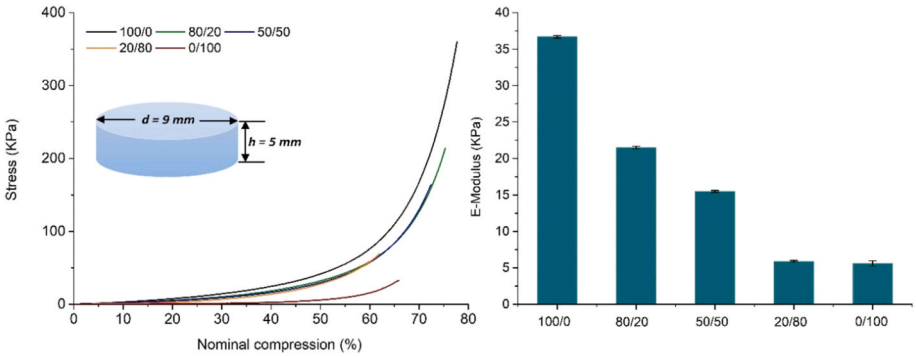

Fig. 5. Compression tests of 10 wt % PEG-TA_5-OH_3/HA-$TA_{2.2}$ hydrogels at different ratios. (n = 3).

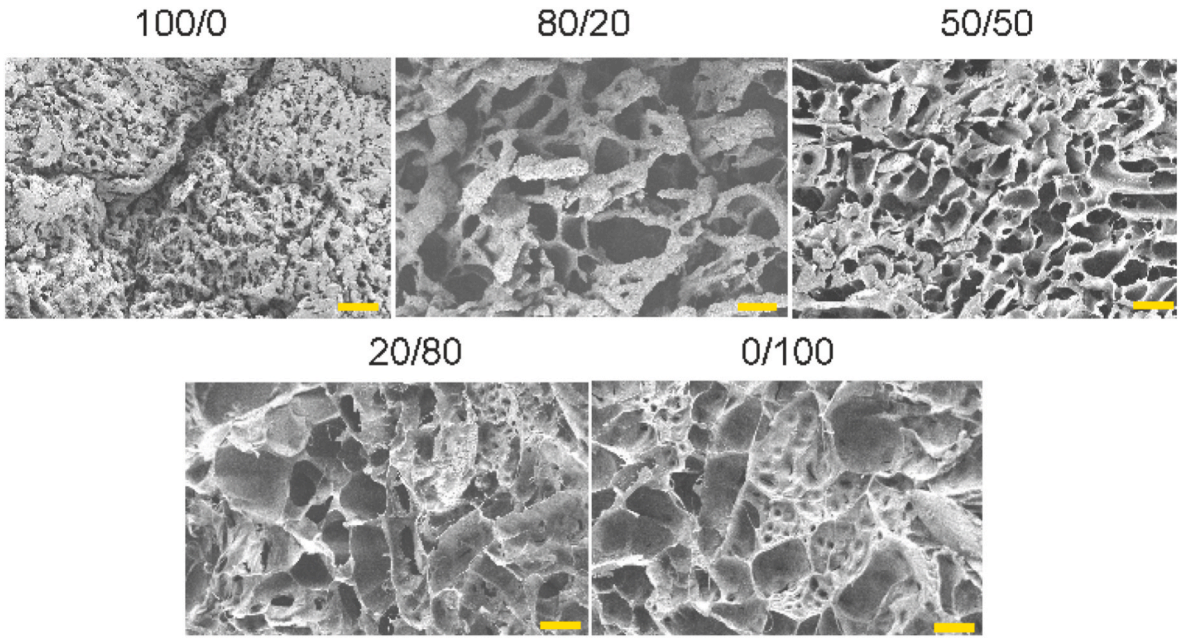

Fig. 6. SEM images of PEG-TA_5-OH_3/HA-$TA_{2.2}$ hydrogels at various weight ratios. Scale bar: 10 μm.

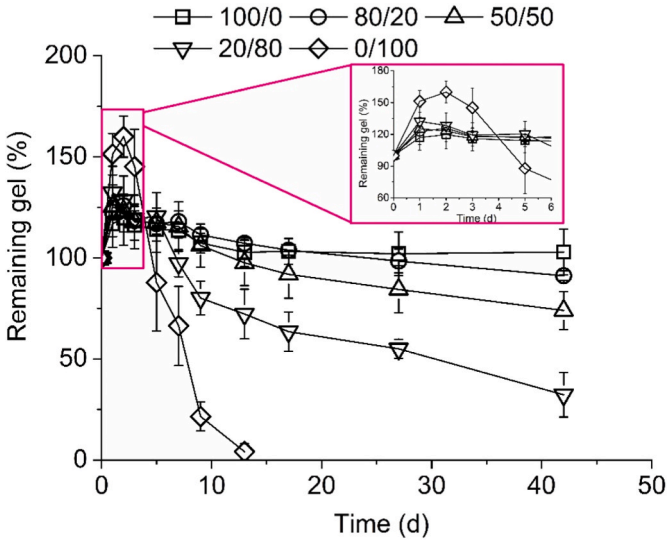

Fig. 7. Enzymatic degradation of 10 wt% PEG-TA_5-OH_3/HA-$TA_{2.2}$ hydrogels in PBS containing 100 U/mL HAse at 37 °C (n = 4).

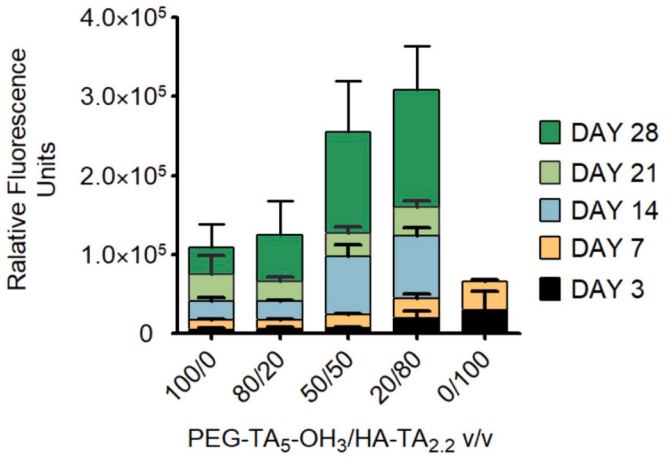

Fig. 8. Metabolic activity of hMSCs encapsulated in PEG-TA_5-OH_3/HA-$TA_{2.2}$ hydrogels with weight ratios of 100/0 to 0/100% at a concentration of 10 wt% (PrestoBlue assay). Cell seeding density: 5 × 10^6 cells/mL. *Note: HA-$TA_{2.2}$ hydrogels fully degraded after 10 days in culture.*

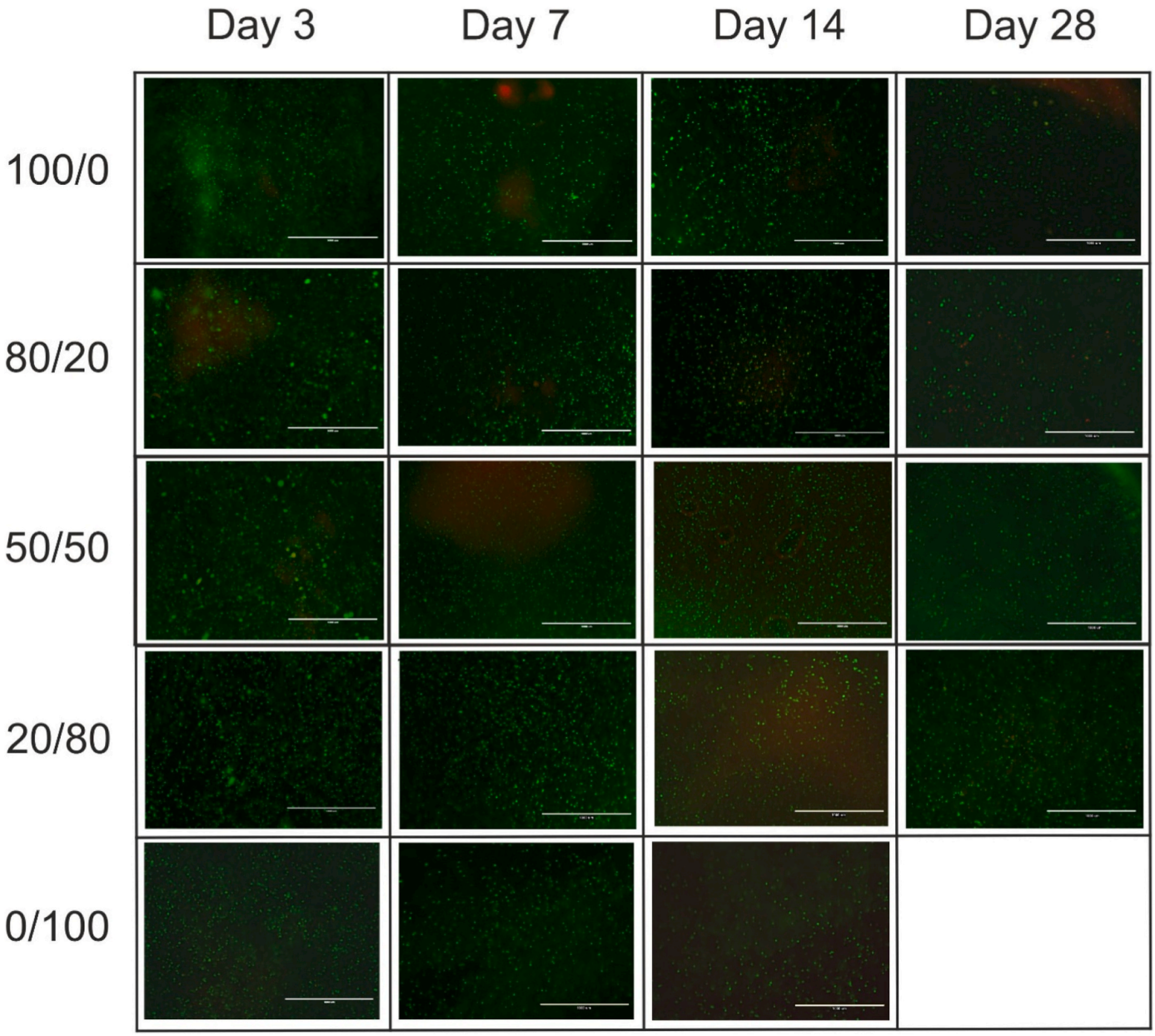

Fig. 9. Live-dead assay showing hMSCs incorporated in PEG-TA_5-OH_3/HA-$TA_{2.2}$ hydrogels with weight ratios of 100/0 to 0/100 at a concentration of 10 wt% after 3, 7, 14 and 21 days in culture. Cell seeding density: 5×10^6 cells/mL. Scale bar: 1000 μm.

measured due to the short gelation times. All hydrogels composed of PEG-TA_5-OH_3/HA-$TA_{2.2}$ mixture showed high storage moduli, at least one order of magnitude higher than a hydrogel prepared from HA-$TA_{2.2}$ at the same HRP and H_2O_2 concentration. The storage moduli (G′) and loss moduli (G″) values graphically presented in Fig. 4B, revealed that highly elastic hydrogels were formed at different PEG-TA_5-OH_3/HA-$TA_{2.2}$ polymer ratios at very low enzyme and H_2O_2 concentrations.

Strain and frequency sweeps were subsequently performed. Using dynamic oscillation testing, an increasing cyclic strain ranging from 0.1 to 50% was applied at a frequency of $\omega_0 = 1.0$ Hz (Fig. 4C). Hydrogels with higher PEG-TA_5-OH_3 content preserve a constant high modulus up to 20% strain, but the modulus rapidly drops at higher strains. These results show that hydrogels comprising PEG-TA_5-OH_3 are highly elastic but cannot resist large deformations. The magnitude of viscoelastic response elicited by a polymeric network is governed primarily by both the length and flexibility of the polymer chains [45–48]. Frequency sweeps from 0.1 to 100 Hz were conducted within the linear viscoelastic region at a strain amplitude γ of 1%. These measurements showed no decrease in the G′ moduli of PEG-TA_5/HA-$TA_{2.2}$ hydrogels and revealed a solid-like behavior over the entire frequency range (Fig. 4D).

3.4. Unconfined compressive modulus

Because the stiffness, from a tissue engineering perspective, has an important impact on cell function [49,50], compression tests were used to examine the compressive modulus of the hydrogels. Hydrogels (10 wt %) containing various weight ratios of PEG-TA_5-OH_3 and HA-$TA_{2.2}$ were pre-formed in PDMS molds and compressed up to failure. The stress-strain curves as depicted in Fig. 5, revealed a highest modulus of 36.7 kPa for the PEG-TA_5-OH_3 hydrogel. Increasing the HA-$TA_{2.2}$ content, the Young's modulus decreased and the PEG-TA_5-OH_3/HA-$TA_{2.2}$ with a ratio of 20/80 (w/w) yielded a value of 5.9 kPa, similar to that of the pure HA-$TA_{2.2}$ hydrogel. This attributes to the increased hydrogel swelling (Fig. 3), representing a lower network density. This resulted in

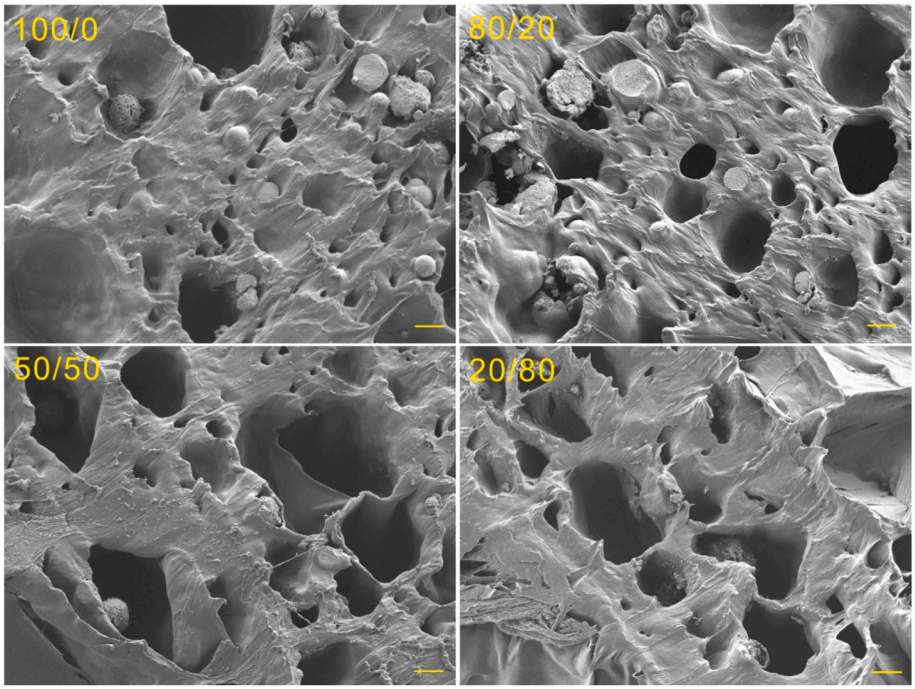

Fig. 10. SEM image of hMSCs incorporated in a PEG-TA_5-OH_3/HA-$TA_{2.2}$ at 100/0, 80/20, 50/50 and 20/80 hydrogels (10 wt%) at day 28 in culture. Scale bar: 10 μm.

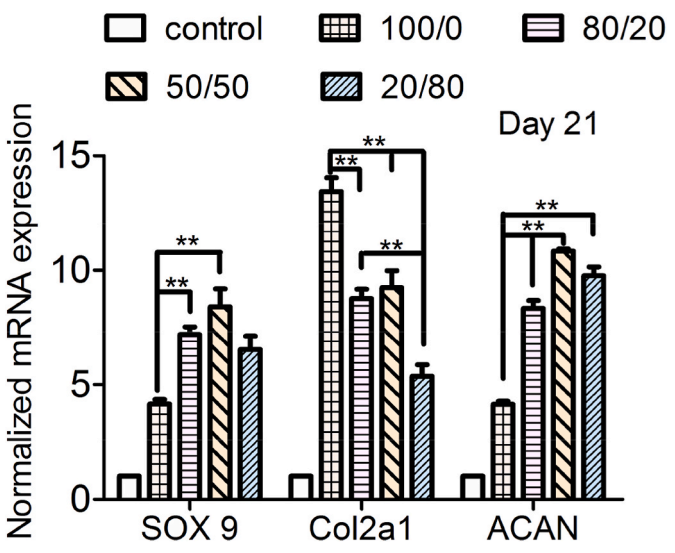

Fig. 11. Gene expression profile of differentiating human chondrocytes at day 21 in culture. Expression of the specific cartilage makers Sox 9, Col2a1 and ACAN in 10 wt% PEG-TA_5-OH_3/HA-$TA_{2.2}$ hydrogel (100/0–20/80) constructs are presented as fold change relative to mRNA expression from a control (cells cultured on tissue plate). Data are shown as mean ± SD (n = 3).

lower values of the elastic modulus and macroscopically softer gels at higher HA-$TA_{2.2}$ weight ratios.

3.5. Hydrogel morphology

The morphology and pore size of the hydrogels were determined by high resolution imaging. SEM images (Fig. 6) of cross-sections of lyophilized hydrogels showed that the PEG-TA_5-OH_3 hydrogel had a lower porosity and a more compact structure. This lower porosity may be due to efficient crosslinking resulting in the highest gel content and lowest water uptake compared to the other compositions as presented in Fig. 3. A HA-$TA_{2.2}$ (100%) gel had the highest pore size whereas at intermediate ratios the PEG-TA_5-OH_3/HA-$TA_{2.2}$ (80/20 and 50/50) hydrogels showed intermediate porosities (Fig. 6).

3.6. In vitro enzymatic degradation

Degradation of scaffolds during tissue regeneration is a basis for remodeling and morphogenesis to form functional tissue. Hyaluronic acid can be degraded via enzymatic hydrolysis using hyaluronidase (HAse) [51]. The degradation of PEG-TA_5-OH_3/HA-$TA_{2.2}$ hydrogels at different polymer ratios was determined by placing PBS containing 100 U/mL HAse on top of 0.2 mL hydrogels. The degradation at 37 °C was monitored by determining the remaining weight as a function of incubation time (Fig. 7). The HA-$TA_{2.2}$ hydrogels showed the highest swelling (up to 165%) in the first 3 days and were fully degraded within 2 weeks. Contrary, PEG-TA_5-OH_3 hydrogels did not show degradation over prolonged periods of time. Hydrogels composed of PEG-TA_5-OH_3 and HA-$TA_{2.2}$ showed an intermediate degradation rate. After 6 weeks, the 10 wt% PEG-TA_5-OH_3/HA-$TA_{2.2}$ hydrogels with weight ratios of 20/80, 50/50 and 80/20 had 30, 75 and 96% remaining gel, respectively. Control experiments of gels in buffer without enzymes were carried out simultaneously showing similar swelling during the first 3 days, but no degradation over a 42-day period (data not shown).

3.7. Cytotoxicity and morphology

In situ forming hydrogels allow an easy encapsulation of cells in the gel matrix. Human mesenchymal stem cells (hMSCs) in proliferation medium were mixed with a hydrogel precursor solution containing the enzyme HRP. These mixtures were subsequently mixed with a hydrogen peroxide solution to give hydrogels with a final polymer concentration of 10 wt%. These hydrogel/cell constructs were analyzed for their cell cytotoxicity by a PrestoBlue assay and a live-dead assay for up to 28 days. The PB assay as presented in Fig. 8, revealed that cells encapsulated in hydrogel constructs of different composition were metabolic active over the entire period. The increase in metabolic activity with increasing HA-$TA_{2.2}$ content is in line with the results of Kawasaki et al.

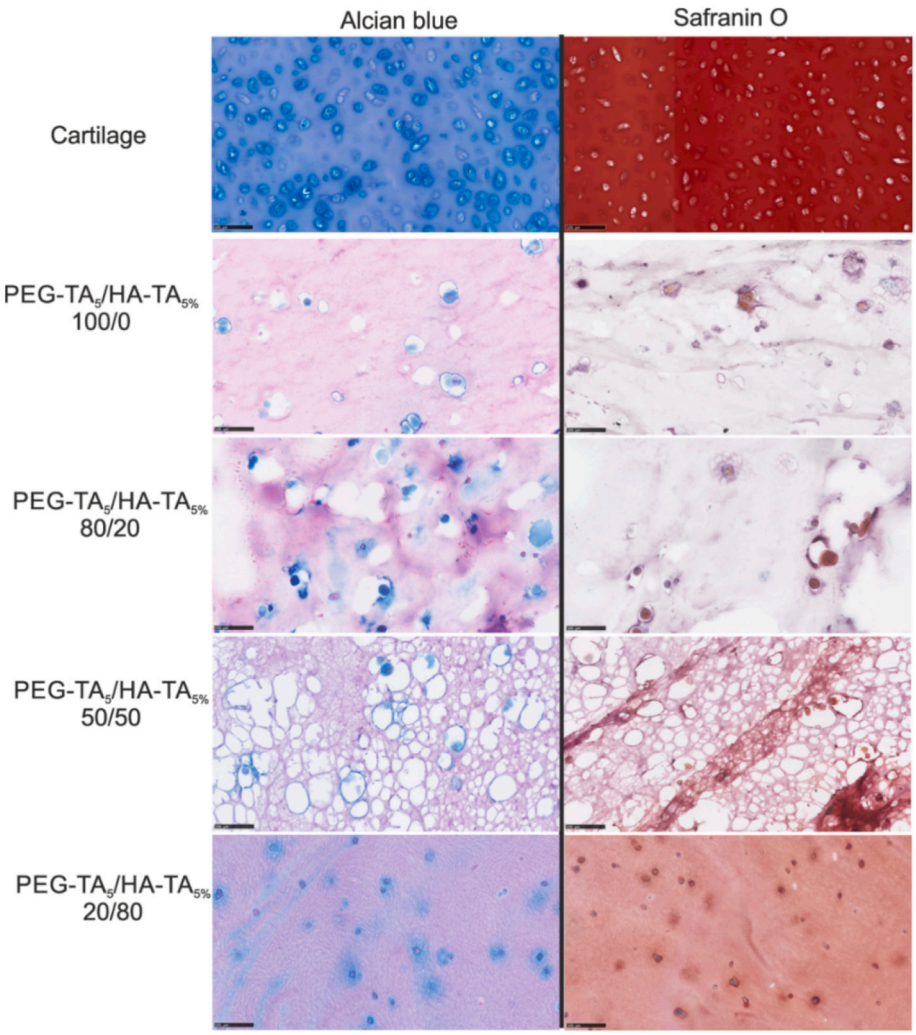

Fig. 12. Alcian blue and safranin O staining of PEG-TA_5-OH_3/HA-$TA_{2.2}$ hydrogels with weight ratios of 100/0 to 0/100 after culturing for 21 days. Scale bar: 100 μm.

who showed that chondrocyte cell numbers were increasing with HA content in collagen gels [52]. The HA-$TA_{2.2}$ hydrogel was degraded after 10 days in culture; therefore, no data was shown afterwards. The biocompatibility of these hydrogels was analyzed by a live-dead assay after culturing for 3, 7, 14 and 21 days, in which the live cells fluoresce green, and dead cells fluoresce red. Merged images with both green and red showed that over 95% of the cells fluoresced green after cultured 21 days, indicating cytocompatible enzymatic crosslinking conditions (Fig. 9).

A typical SEM image of a hydrogel cross-section of encapsulated hMSCs in a PEG-TA_5-OH_3/HA-$TA_{2.2}$ 50/50 at day 28 is shown in Fig. 10. The cells encapsulated inside the gel retained a round shape for 28 days in culture, which indicates that the hydrogels were biocompatible and support cell survival but do not stimulate cell spreading.

3.8. In vitro differentiation of human chondrocytes encapsulated in hydrogels

Differentiation of human chondrocytes was quantitatively evaluated using RT-PCR. The ability of chondrocytes encapsulated in the hydrogels, to maintain a chondrocytic phenotype and form cartilaginous tissue in vitro in chondrogenic differentiation medium was followed up to 21 days. Gene expression of SOX 9, collagen type II and aggrecan as specific chondrogenic markers was analyzed and results are presented in Fig. 11. The gene expression was normalized to control samples in which cells were cultured on tissue culture plastic (2D). As shown in Fig. 11, chondrocytes in hydrogels (3D) expressed significantly higher levels of the markers compared to cells cultured in 2D, similar as reported by Discher et al. and Yang et al. [50,53].

The expression levels of these three genes depended on the hydrogel composition. Chondrocytes encapsulated in PEG-TA_5-OH_3/HA-$TA_{2.2}$ 50/50 hydrogels showed significantly upregulated SOX 9 and ACAN gene expressions compared to those in PEG-TA_5-OH_3 hydrogels ($p < 0.01$). However, PEG-TA_5-OH_3 hydrogels expressed higher collagen type II levels than all other hydrogels, indicating an existence of a chondrocyte phenotype. The results showed that at higher HA content SOX9 and ACAN levels were upregulated in an inverse relation with the mechanical strength of the hydrogel. Our data also suggest a direct correlation between collagen type II mRNA expression levels and gel mechanical properties. An increased expression of Collagen type II is observed for hydrogels with higher storage modulus [50]. Additionally, the accumulation of a chondrogenic specific ECM in the hydrogel/cell constructs was examined by histological analysis. Bovine cartilage sections were stained as positive control. Alcian blue and Safranin O, which stain glycosaminoglycan with an intense blue color (Fig. 12, left) and acidic proteoglycans with an orange-red color (Fig. 12, right) were clearly observed in hydrogels after 28 days in culture, showing the production of a cartilage specific ECM. As shown in Fig. 12, GAG and proteoglycan staining were observed in all hydrogels with different compositions. PEG-TA_5-OH_3 hydrogels showed distinct cell encapsulation and less ECM production, which may be due to the high stiffness of the PEG gels and low cell-hydrogel interactions [54]. However, in the

presence of HA-$TA_{2.2}$, intense blue and red were produced. Especially, in the PEG-TA_5-OH_3/HA-$TA_{2.2}$ 20/80 hydrogel, newly formed matrix was evenly distributed over the construct. This may be due to HA which can easily interact with proteins present on the surface of the cells as well as with cells via CD44 cell surface receptors.

4. Conclusion

Biodegradable synthetic/natural hybrid hydrogels were successfully formed by the oxidative coupling reaction of tyramine conjugates of a star shaped 8-arm PEG and hyaluronic acid in the presence of H_2O_2 and HRP. The physicochemical properties of the hydrogels, including gelation time, mechanical properties, swelling ratio and degradation could be adjusted by varying the HRP concentration and polymer composition. Hybrid hydrogels with different ratios of 8-arm PEG and HA showed excellent cytocompatibility. The metabolic activity of hMSCs encapsulated in the hydrogels was highest at a higher HA content of 80%. Moreover, human chondrocytes encapsulated in hydrogels maintained their chondrocyte phenotype and formed a cartilaginous specific matrix. High collagen type II expression was observed in higher elastic gels. Interestingly, higher SOX9 and ACAN expression was present in gels with higher HA content resulting in gels with lower stiffness. These results demonstrate that PEG-TA/HA-TA hybrid hydrogels are promising candidates for tissue regeneration applications.

CRediT authorship contribution statement

Rong Wang: Formal analysis, Investigation, Writing – original draft, Visualization. **Xiaobin Huang:** Investigation. **Bram Zoetebier:** Validation, Writing – review & editing. **Pieter J. Dijkstra:** Writing – review & editing, Funding acquisition. **Marcel Karperien:** Writing – review & editing, Funding acquisition, Supervision.

Declaration of competing interest

We report no financial interests or potential conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioactmat.2022.05.020.

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