Injectable Hydrogels by Enzymatic Co-Crosslinking of Dextran and Hyaluronic Acid Tyramine Conjugates

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Summary: In this study, injectable hydrogels were prepared by the horseradish peroxidase mediated co-crosslinking of dextran-tyramine (Dex-TA) and hyaluronic acid-tyramine (HA-TA) conjugates intended for cartilage tissue engineering. In general the gelation times of 10 wt% polysaccharide solutions are $<$ 20 seconds and their storage moduli can be adjusted by varying the composition between Dex-TA and HA-TA. Dex-TA/HA-TA (50/50) hydrogels were fully degradable in the presence of hyaluronidase. Chondrocytes incorporated in 10 wt% Dex-TA and Dex-TA/HA-TA (50/50) gels showed good viability after 28 days. These results indicate that Dex-TA/HA-TA (50/50) hydrogels are promising injectable and biodegradable hydrogels for cartilage repair.

Keywords: biomaterials; crosslinking; enzymes; hydrogels; polysaccharides

Introduction

Tissue engineering represents a promising method to regenerate damaged cartilage.[1] This method involves the incorporation of cells (chondrocytes) into a scaffold, which serves as a temporary extracellular matrix (ECM) and promotes cartilage regeneration. *In situ* forming hydrogels are attractive scaffold candidates because they allow minimally invasive techniques, are able to fill irregularly shaped defects, and easy encapsulation of chondrocytes in a polymer solution is possible prior to gelation.[2–5] Additionally, such hydrogels provide a 3D environment, which facilitates cells proliferation and differentiation and meanwhile allows transport of nutrients and waste products. Because hydrogels are 3D elastic networks having high water content, they mimic hydrated native cartilage tissue and are considered suitable scaffolds for cartilage tissue engineering.

Injectable hydrogels can be obtained via chemical crosslinking, for example photopolymerization. In this approach, a solution of a polymer containing vinylic groups converts into a gel by exposure to visible or ultraviolet light in the presence of photoinitiators.[6–9] The preparation of hydrogels by photo-initiation has the advantage of fast crosslinking rates upon exposure to UV irradiation. However, the disadvantage of this method is the exposure of cells to UV at high intensities or long exposure times which may have an adverse effect on cellular metabolic activity.[10] Moreover, local heat release during the crosslinking process may give rise to cellular necrosis.[11] Alternatively injectable hydrogels can be generated via Michael type addition reactions of thiol groups to (meth)acrylate, (meth)-acrylamide, or vinyl sulfone groups.[12–17] In this approach, it was also shown that thiol-bearing bioactive molecules such as adhesion peptides and matrix metalloproteinase substrate peptides can be relatively easily incorporated to create biomimetic hydrogels.[14] Generally, hydrogels that are prepared via Michael type addition reactions have gelation times less than 0.5 min to about

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1 hour, and have moderate storage moduli and their properties can be adjusted by tuning the reactivity of functional groups and crosslinking density.[12,13,17,18] Since Michael additions reactions generally take place at mild conditions, the reaction does not seriously influence cell viability during the hydrogel formation process. It was reported that incorporated cells in these type of hydrogels remained viable and survived from days to months, depending on the applied materials.[19,20] However, some caution has to be taken in the use of thiol functional groups for unreacted thiols may cause cell death.[21]

Recently, we showed that injectable hydrogels can be prepared by the enzymatic crosslinking of polysaccharide tyramine conjugates.[22–25] Crosslinking takes place via an oxidative coupling reaction of phenol moieties in the presence of horseradish peroxidase (HRP) and H_2O_2.[26] This method afforded fast gelation and showed good cytocompatibility of incorporated chondrocytes.

Hyaluronic acid has abundant carboxylic acid groups, amendable to various types of chemical modifications. This offers the opportunity to conjugate hyaluronic acid with tyramine groups by activation of the carboxylic acid groups and subsequent reaction with the tyramine amine groups. In this way, biofunctional scaffolds may be created to modulate cell adhesion, migration, proliferation and differentiation as well as new tissue formation.

We here describe biofunctional injectable hydrogels based on dextran and hyaluronic acid applicable as injectable hydrogels for cartilage tissue engineering. The properties of the hydrogels such as gelation times, gel content, water uptake and mechanical properties were investigated. Moreover, bovine chondrocytes were incorporated in these hydrogels to evaluate their cytocompatibility.

Materials and Methods

Materials

Dextran ($M_w = 15$ to 25 kg/mol) was obtained from Sigma-Aldrich and dried by lyophilization. Hyaluronic acid sodium salt ($M_w = 15$ to 25 kg/mol) was purchased from CPN-shop. N-hydroxysuccinimide (NHS), tyramine and p-nitrophenyl chloroformate (PNC), Anhydrous Dimethylformamide (DMF), Pyridine (anhydrous), hydrogen peroxide (H_2O_2) and lithiumchloride, Horseradish peroxidase (HRP, 311 purpurogallin unit/mg solid) were obtained from Sigma-Aldrich and were used without further purification. N-Ethyl-N′-(3-dimethylaminopropyl) carbodiimide hydroxide (EDAC) was obtained from Fluka. Phospate buffered saline (PBS, pH 7,4) was purchased from B. Braun Co. Dextran-tyramine conjugates (Dex-TA) was synthesized as described previously.[25]

Synthesis

Hyaluronic Acid-Tyramine Conjugate (HA-TA)

A HA-TA conjugate was synthesized by the coupling reaction of primary amine groups from tyramine to hyaluronic acid carboxylic groups using EDAC/NHS activation. Sodium hyaluronate (2 g) was dissolved in 30 mL of MES buffer (0.1 M, pH 6.0), to which EDAC (288 mg, 1.5 mmol) and NHS (227 mg, 1.5 mmol) were added. After 30 min of stirring 6 mL of a DMF solution containing tyramine (69 mg, 0.5 mmol) was added and the mixture was stirred under nitrogen for 3 days. The mixture was neutralized with 1 M NaOH and ultrafiltrated (MWCO 1000), first with PBS and then MilliQ-water. The resultant HA-Tyr conjugate was obtained after lyophilization as a white foam. Yield: 1.89 g (95%). DS (UV/Vis): 17.

Characterization

^{1}H-NMR

^{1}H (300MHz) NMR spectra were recorded using a Varian Inova NMR spectrometer. Polymers were dissolved in D_2O or DMSO at a concentration of 0.020 g/ml.

UV/Vis

The degree of substitution of tyramine residues in the HA-TA conjugate was 17 as determined by an UV measurement. The HA-TA conjugate was dissolved in PBS at a concentration of 5 μg/mL and the absorbance was measured at 275 nm using a Cary 300 Bio

spectrometer (Varian). The absorbance was correlated to the DS of tyramine groups in the HA-TA using a calibration curve from tyramine in PBS.

Hydrogel Formation and Gelation Time

Hydrogel samples of Dex-TA and Dex-TA/HA-TA (50/50) at a polymer concentration of 10 or 20 wt% were prepared in vials at room temperature. In a typical procedure, to a polymer solution (200 μL, 12.5 wt% and 25 wt% respectively in PBS), a freshly prepared solution of H_2O_2 (25 μL of 0.3% stock solution in PBS) and HRP (25 μL of 150 unit/mL stock solution in PBS) were added and the mixture was gently shaken until gel formation occurred. The time to form a gel (denoted as gelation time) was determined using the vial tilting method. No flow within 1 minute upon inverting the vial was regarded as the gel state. The experiment was performed in triplicate.

Gel Content and Degree of Swelling

To determine the gel content, samples of 25 mg or 50 mg (W_d) of Dex-TA or Dex-TA/HA-TA (50/50) were converted into hydrogels (10 and 20 wt%) as described above. The samples were subsequently incubated in 3 mL of MilliQ water and the solution was refreshed every day for 3 days to remove salts and uncrosslinked polymer. The samples were dried in a vacuum oven to a constant weight (W_g). The gel content was expressed as $W_g/W_d \times 100\%$.

The degree of swelling of the hydrogels was determined as follows. 250 μL hydrogels were weighted (W_i) and then immersed in 3 mL of MilliQ water for 3 days to reach swelling equilibrium. The swollen samples were removed from the water and after removal of surface water, the samples were weighted (W_s). the degree of swelling was calculated from: $(W_s\text{-}W_i)/W_i$. The experiments were performed in triplicate.

Rheological Analysis

Rheological experiments were carried out with a MCR 301 rheometer (Anton Paar) using parallel plates (25 mm diameter, 0°) configuration at 37 °C in the oscillatory mode. In a typical experiment the polymer dissolved in PBS (200 μL, 12.5 and 25 wt% polymer) was placed at the ground plate and a freshly mixed solution of HRP (25 μL, 150 units/mL stock solution) and H_2O_2 (25 μL, 0.3% stock solution) was pipetted into the polymer solution. After the samples were mixed, the upper plate was immediately lowered to a measuring gap size of 0.3 mm, and the measurement was started. To prevent water evaporation, a layer of oil was introduced around the polymer sample. The evolution of the storage (G′) and loss (G″) modulus was recorded for 6 hours, using a strain of 0.1% and a frequency of 0.5 Hz.

Swelling and Enzymatic Degradation Assays of Hydrogels

Hydrogel samples (250 μL) were prepared in vials according to the procedure described above. Samples were taken out of the vials, accurately weighed (W_i), and incubated in 3 mL of PBS containing 20 U/mL hyaluronidase at 37 °C. The enzyme-solution was replaced every day during the first 3 days, then twice a week. The hydrogels were weighted (W_t) and the remaining gel (%) was calculated from the initial weight (W_i) and remaining gel weight after exposure to the enzyme containing buffer (W_t), expressed as $W_t/W_i \times 100\%$. The experiments were performed in triplicate.

In Situ Chondrocyte Incorporation

Bovine chondrocytes were isolated as previously reported.[22–24] Hydrogels containing chondrocytes were prepared under sterile conditions by mixing a polymer/cell suspension with HRP/H_2O_2. Polymer solutions of Dex-TA and Dex-TA/HA-TA (50/50) were made in Chondrocyte expansion medium (DMEM with 10% heat inactivated fetal bovine serum, 1% penincilin/streptomycin (Gibco), 0.01 M MEM nonessential amino acids (Gibco), 10 mM HEPES and 0.04 mM L-proline) and HRP and H_2O_2 stock solutions were made in PBS. All components were sterilized by filtration through filters with a pore size of 0.22 μm. Chondrocytes (P0) were dispersed in the polysaccharide precursor solution. The

hydrogels were prepared using the same procedure as in the absence of cells. The final polymer concentration was 10 wt% and the cell seeding density in the gels was 20×10^6 cells/mL. After gelation, the hydrogels (100 μL each) were transferred to a culture plate and 2 mL of chondrocyte differentiation medium (DMEM with 0.1 μM dexamethasone (Sigma-Aldrich), 100 μg/mL sodium pyruvate (Sigma-Aldrich) 0.2 mM ascorbic acid, 50 mg/mL insulin-transferrin-selenite (ITS + 1, Sigma-Aldrich) 100 U/ml penicillin, 100 μg/ml streptomycin, 10 ng/mL transforming growth factor ß3 (TGF-ß3, R&D systems)) 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

A viability study on the hydrogel-mixtures with incorporated cells was performed with a live-dead assay. For the live-dead assay at day 14 and 28 the hydrogel constructs were rinsed with PBS and stained with calcein AM/ethidium homodimer using the Live/Dead assay Kit (Invitrogen), according to the manufactures instruction. Hydrogel/cell constructs were visualized using fluorescence microscopy (Nikon Eclipse E600), as a result living cells fluorescence green and the nuclei of dead cells red.

Scanning Electron Microscopy (SEM)

The morphology of the chondrocytes in the hydrogels was studied using a Philips XL 30 ESEM-FEG scanning electron microscope operating at a voltage of 5 or 10 kV. After 14 and 28 days *in vitro* culturing the hydrogel/cell constructs were fixed with formalin by sequential dehydration and critical point drying. These samples were gold sputtered (Carringdon) and analyzed with SEM.

Results and Discussion

Synthesis

In this study, dextran-tyramine (Dex-TA) conjugates were prepared by first activating hydroxyl groups of dextran with p-nitrophenyl chloroformate (PNC) to form p-nitrophenyl carbonate derivitaves (Dex-PNC). Subsequently, the obtained Dex-PNC was treated with an excess of tyramine to give Dex-TA conjugates. The structure of the Dex-TA conjugate was confirmed by ^{1}H-NMR and the degree of substitution (DS) of TA to the dextran was 15 (Figure 1). The DS is defined as the number of conjugated groups per 100 anhydroglucose units in dextran.

The key material of this study, a hyaluronic acid-tyramine (HA-TA) conjugate, with a degree of substitution of tyramine residues of 17 (DS 17), was synthesized by the coupling reaction of the amino group of tyramine to the carboxylic acid groups of hyaluronic acid using EDAC/NHS activation (Figure 1).

Hydrogel Formation and Characterization

Hydrogels were prepared by the horseradish peroxidase (HRP, 15 units·mL^{-1}) and H_2O_2 (0.01 M) mediated coupling reaction of phenolic moieties in Dex-TA (DS 15) and HA-TA (DS 17) conjugates in PBS. Coupling of phenolic rings through the radicals generated at the ortho positions of the phenolic groups can both take place via carbon-carbon bond and carbon-oxygen bond formation. In previous research,[22–25] it was shown that at these concentrations of HRP and H_2O_2 the resulting hydrogels/cell constructs have good cytocompatibility.[24] Dex-TA and Dex-TA/HA-TA (50/50) hydrogels were prepared at polymer concentrations of 10 and 20 wt%.

Because a short gelation time is a prerequisite for injectable gel/cell constructs, the gelation times of Dex-TA and Dex-TA/HA-TA (50/50) were investigated by the vial tilting method (Figure 2A). The enzymatic crosslinking of Dex-TA 10 and 20 wt% hydrogel led to fast gelation of approximately 10 seconds. The gelation time of a Dex-TA/HA-TA (50/50) at 10 wt% was also ca. 10 seconds but at 20 wt% the gelation time increased considerably to 60 seconds. At this concentration a pronounced increase in viscosity was

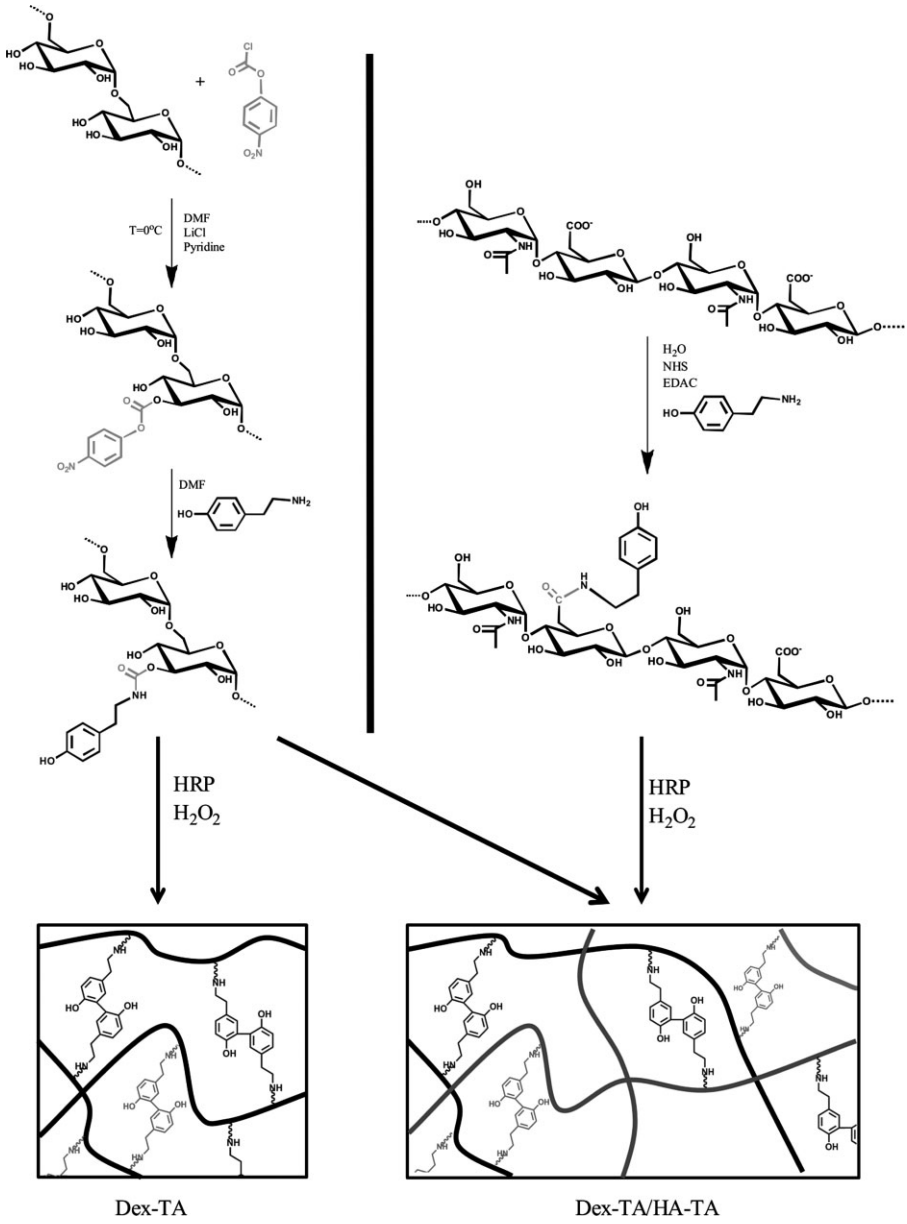

Figure 1.
Synthesis of Dex-TA conjugates and HA-TA conjugates and HRP/H_2O_2 mediated crosslinking to Dex-TA or Dex-TA/HA-TA (50/50) hydrogels.

observed. This increase in viscosity likely hampers the diffusion of HRP and H_2O_2 and tyramine units resulting in a much slower crosslinking rate.

The gel content of the crosslinked hydrogels was determined gravimetrically by extracting the gels with water for 3 days and was approximately 80% for all gels prepared (Figure 2B).

The Dex-TA/HA-TA (50/50) hydrogels show a much higher degree of swellling compared to Dex-TA hydrogels (Figure 2C).

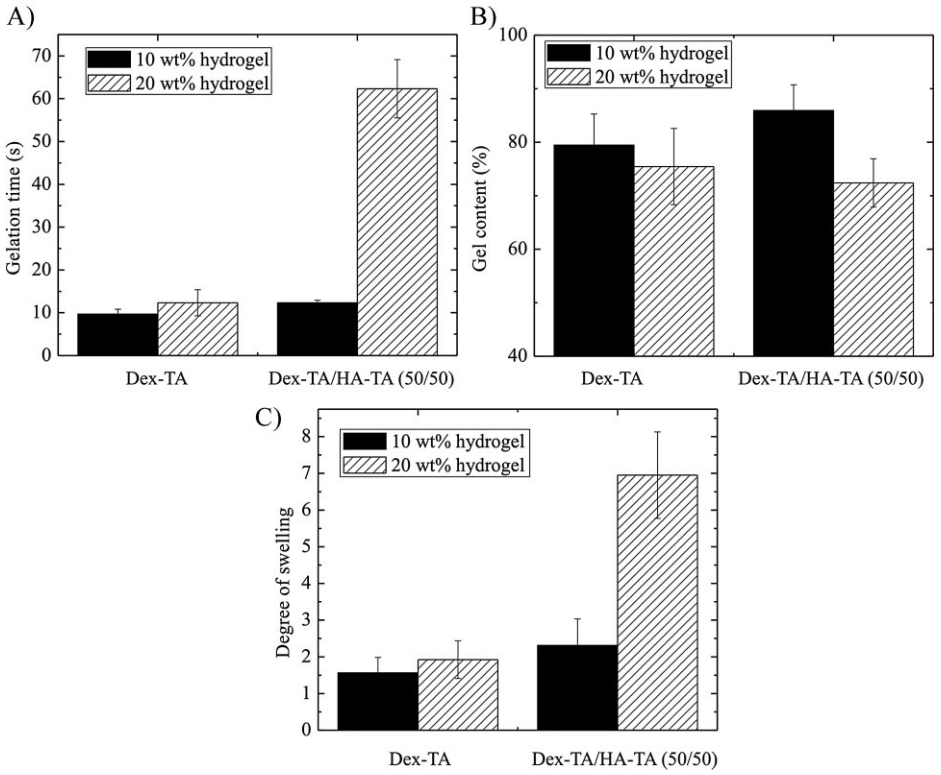

Figure 2.
A) Gelation times of 10 and 20 wt% hydrogels. B) Gel content of 10 wt% and 20 wt% hydrogels. C) Degree of swelling of 10 wt% and 20 wt% hydrogels.

Especially at 20 wt% a lower crosslink density due to the high viscosity may hamper efficient crosslinking.

Rheological Analysis

The mechanical properties of 10 and 20 wt% Dex-TA and Dex-TA/HA-TA (50/50) hydrogels were studied by oscillatory rheology experiments at 37 °C. Gel formation kinetics was followed by monitoring the storage modulus (G′) and loss modulus (G″) in time directly after mixing a polymer and a HRP/H_2O_2 solution. It was seen that after 3 minutes all samples reached a plateau value. When the plateau was reached some notable differences could be seen (Figure 3). For Dex-TA 10 and 20 wt% hydrogels the stiffness is approximately the same. The Dex-TA/HA-TA (50/50) hydrogels showed a decrease in the stiffness upon increasing the polymer concentration from 10 to 20 wt%. This is in line with the results found for the gel content and water uptake. The low crosslink density makes the 20 wt% Dex-TA/HA-TA (50/50) difficult to handle and only the 10 wt% Dex-TA and Dex-TA/HA-TA (50/50) hydrogels were used to test the degradation and cytocompatibility.

Comparing the 10 wt% Dex-TA and Dex-TA/HA-TA (50/50) hydrogels it is seen that addition of HA-TA increases the stiffness of the hydrogel. Whereas 10 wt% Dex-TA hydrogels have tan δ values of 0.18 incorporation of HA-TA lower this to 0.13 in Dex-TA/HA-TA (50/50) hydrogels.

Degradation

The enzymatic degradation profiles of the 10 wt% Dex-TA and Dex-TA/HA-TA (50/50) were determined by placing 3 mL of

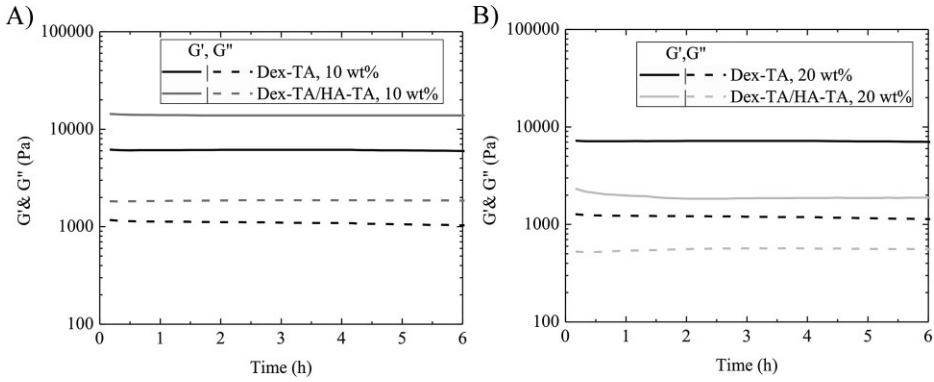

Figure 3.
Storage and loss modulus of 10 wt% Dex-TA and Dex-TA/HA-TA (50/50) hydrogels.

PBS containing 20 $U \cdot mL^{-1}$ hyaluronidase (6 times as much as present in human articular cartilage[27]) on top of 250 μL of hydrogels. The hydrogels were kept at 37 °C and their wet weights were monitored at regular time intervals. The remaining gel (%) was expressed as the gel weight after exposure to enzyme buffer (W_t) divided by the original gel weight after preparation (W_i). In buffer without enzymes present, the Dex-TA and Dex-TA/HA-TA (50/50) hydrogels swelled and the weight increased during the first 3 days, and remained stable up to 50 days (data not shown). In the presence of hyaluronidase, the Dex-TA showed no degradation for 60 days. In the first days the weight of the Dex-TA/HA-TA (50/50) increased because of chain scission of HA causing increased water uptake, and followed by weight loss due to the dissolution and release of small fragments. It was found that the Dex-TA/HA-TA (50/50) was fully degradable within 55 days (Figure 4). Compared to previously reported HA-TA hydrogels[28] which were completely degraded within 1 day in the presence of 25 $U \cdot mL^{-1}$ of hyaluronidase in PBS, these gels were much more stable most likely due to co-cross-linking with Dex-TA. Compared to results of HA grafted with Dex-TA obtained by Jin et al. it is seen that combining Dex-TA and HA-TA gives similar stabilization of the hydrogels, but avoids the relative complex synthesis of the HA grafted with Dex-TA.[23]

Cell Viability and Proliferation

The cytocompatibility of 10 wt% Dex-TA and Dex-TA/HA-TA (50/50) was analyzed by a live/dead assay after culturing for 14 and 28 days, in which the live cells fluorescence green, and dead cells fluorescence red. For both the Dex-TA and Dex-TA/HA-TA (50/50) it was shown that 95% of the cells fluorescence green after both 14 (data not shown) and 28 days (Figure 5).

It is known that chondrocytes in culture may rapidly dedifferentiate and obtain a fibroblast–like phenotype.[29] Whereas

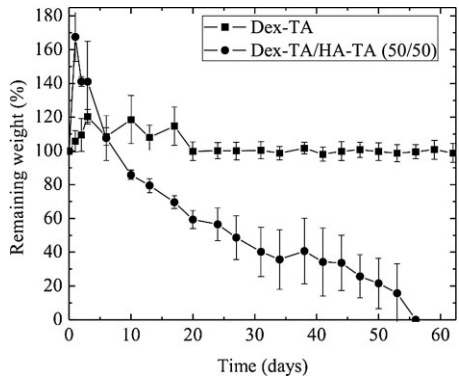

Figure 4.
Enzymatic degradation of Dex-TA and Dex-TA/HA-TA (50/50) hydrogels in PBS containing 20 $U \cdot mL^{-1}$ hyaluronidase at 37 °C.

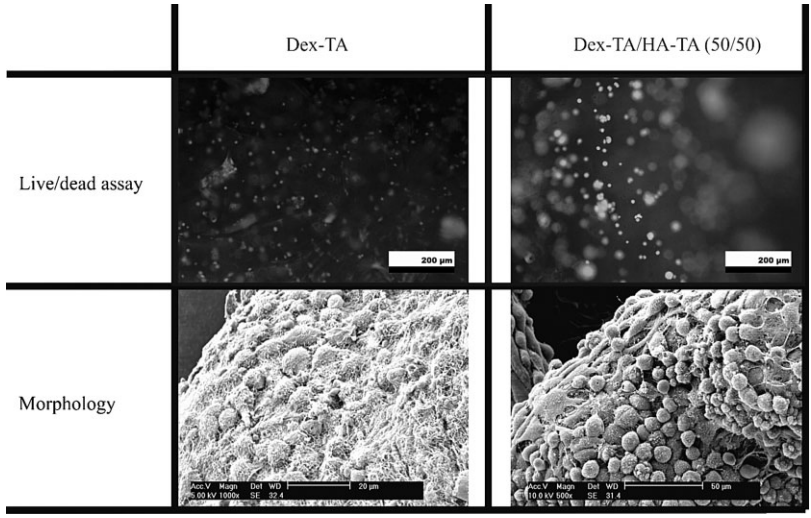

Figure 5.
Live-dead assay showing the chondrocytes incorporated in Dex-TA or Dex-TA/HA-TA (50/50) after 28 days. SEM images showing the morphology of chondrocytes incorporated in Dex-TA or Dex-TA/HA-TA (50/50) hydrogels at 28 days.

these dedifferentiated show a flattened morphology a round cell shape is correlated with the maintenance of the chondrocyte phenotype. To assess if the chondrocytes maintained a round shape SEM images were taken of Dex-TA and Dex-TA/HA-TA (50/50) hydrogels cultured for 28 days. The morphology of the chondrocytes incorporated in the Dex-TA hydrogel showed a flattened morphology. Contrary, the Dex-TA/HA-TA (50/50) hydrogels exhibited a distinctly round cell shape showing that hyaluronic acid may stimulate the regeneration of cartilage in these constructs (Figure 5).

Conclusion

We have shown that injectable hydrogels containing naturally occurring hyaluronic acid, can be prepared via enzymatic crosslinking of Dex-TA and HA-TA conjugates. The gelation is fast with gelation times lower than 1 min, which can be regulated by varying the polymer concentration. Hydrogels containing HA-TA are readily degraded in the presence of hyaluronidase. 10 wt% Dex-TA/HA-TA (50/50) hydrogels had a higher storage modulus compared to Dex-TA, while 20 wt% Dex-TA/HA-TA (50/50) showed an increase in viscosity hampering the crosslinking reaction. The behavior of chondrocytes incorporated in Dex-TA/HA-TA (50/50) hydrogels demonstrated that the gel systems had good cytocompatibility. These results indicate that Dex-TA/HA-TA (50/50) hydrogels have a high potential as matrices for cartilage tissue engineering.

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