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Self-attaching and cell-attracting in-situ forming dextran-tyramine conjugates hydrogels for arthroscopic cartilage repair

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ABSTRACT

Small cartilage defects are frequently treated with debridement or left untreated, predisposing to early onset osteoarthritis. We propose to fill these defects with a cell-free injectable hydrogel comprising dextran-tyramine conjugates (Dex-TA) that can be applied during arthroscopic procedures. In this study, we report on the adhesion mechanism between cartilage and Dex-TA hydrogels and enhancement of cell ingrowth by incorporation of Heparin-tyramine (Hep-TA) conjugates. The enzyme-catalyzed crosslinking reaction of Dex-TA and Hep-TA hydrogels is based on covalent bonding of hydroxyphenyl residues. We hypothesized that this reaction results in covalent bonding of the hydroxyphenyl residues in Dex-TA and Hep-TA to tyrosine residues in cartilage matrix proteins. The involvement of TA residues was confirmed by modelling the enzymatic reaction occurring during gelation. The mechanical analysis indicated that higher tyramine content led to stronger binding. Interfacial cartilage-hydrogel morphology and Raman spectroscopy demonstrated collagens' reorganization and evidenced the coupling of TA to tyrosine residues in collagen. Moreover, the addition of Hep-TA induced cell recruitment. Collectively, *in vitro* and *ex vivo* functional studies evidenced the covalent bonding of TA-containing hydrogels to tyrosine residues in cartilaginous matrix proteins. Moreover, the cell-attracting ability of these hydrogels could be explored to guide tissue repair in focal cartilage defects, preventing or delaying the onset osteoarthritis.

1. Introduction

Articular cartilage degeneration is part of the clinical condition of osteoarthritis (OA). OA is the most common cause of chronic musculoskeletal pain and mobility disability. A diversity of treatments has the prospective to improve healing of articular surfaces, including micro-fracture of subchondral bone, mosaicplasty, autologous cell implantation (ACI), growth factors, and artificial matrices [1]. However, each of these methods exhibits disadvantages, for instance micro-fracture leads to fibro-cartilage, mosaicplasty results in poor integration and donor site morbidity and with ACI more hyaline cartilage is obtained, yet the long term outcome is still debated [2–5]. The use of an artificial matrix for filling up small focal defects is an attractive strategy for treatment of defects that are otherwise left untreated and may ultimately predispose to early onset osteoarthritis. We hypothesize that by applying a non-cytotoxic and degradable hydrogel directly after the debridement, mechanical stability of cartilage can be improved while facilitating tissue regeneration. Such hydrogel may counteract mechanically induced cartilage erosion and, thus may effectively delay or even prevent the development of osteoarthritis. Ideally, the hydrogel should also be an adhesive material that can be applied in analogy to wound dressings [6].

To translate this to an approach that can be applied during an arthroscopic procedure, the following pre-requisites have to be fulfilled: i) the material should initially be fluid to fill up irregular defects, ii) should improve mechanical stability, iii) preferably mimic cartilaginous matrix properties, and finally iv) should be fully compatible with cell growth and tissue remodelling. More

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importantly, and in addition to these features, the biomaterial must bind strongly to the native tissue, preferably by covalent bonding. Hydrogels consisting of natural polymers are likely to fulfil these requirements, due to their resemblance to the cartilaginous matrix [7–10]. Injectable hydrogels are of particular interest for cartilage tissue engineering, since they can be applied in minimally invasive procedures [11]. Enzymatic crosslinking is an increasingly attractive method to induce *in situ* hydrogel formation due to the mildness of the process. Naturally occurring enzymes such as transglutaminases and peroxidases are commonly used as the catalysts [12–15]. Peroxidases are natural cross-linkers commonly used for enzyme-catalyzed polymerization and are involved in several catalytic processes within the human body. Examples of such enzymes are eosinophil peroxidase, myeloperoxidase and lactoperoxidase [16,17]. We and others have previously reported the introduction of hydroxyphenyl groups in the backbone of naturally occurring polymers, such as dextran, hyaluronic acid and chitosan, which allow *in situ* gelation mediated by non-toxic concentrations of H_2O_2 and horseradish peroxidase. Using this system, covalent bonds between hydroxyphenyl groups are efficiently formed. Hydroxyphenyl residues are also present in tyrosine suggesting that during *in situ* gel formation the polymers may covalently bind to tyrosine-containing extracellular matrix proteins.

Commercially available tissue glues for surgical purposes based on fibrin are the current golden standard. Although non-cytotoxic, these glues tend to rapidly degrade, in particular in the presence of increasing concentrations of chondrocytes [18]. Other injectable adhesive hydrogels based on chitosan show initial fixation, yet are slowly gelating and mechanically fragile [19]. An elegant multi-step system using chondroitin sulphate (CS)-based glue to covalently link cartilage to a hydrogel [20] has been reported. This approach improves the mechanical stability; however, it requires an invasive procedure involving cartilage digestion followed by UV crosslinking. More recently, a scaffold adhesive based on CS and polyethylene glycol (PEG) has been described [21]. Although these CS-PEG gels were able to covalently crosslink to primary amines of collagens by the formation of amide bonds, they were not permissive for cell ingrowth and matrix remodelling. The ability of hydrogels to equally facilitate cell infiltration and matrix production is fundamental to develop a cell-free biomaterial to guide tissue repair. Cell-free systems demand less legislation for FDA approval; consequently, translation into clinical settings can occur more rapidly. Moreover, additional surgeries to collect autologous chondrocytes are avoided, as well as time and resource-consuming cell expansion steps.

Simultaneous positioning of a material at the diseased cartilage surface and cell homing by one simple injection are keys for the success of cartilage repair strategies. Therefore, in this study, we explored the potential of covalent bond formation between the

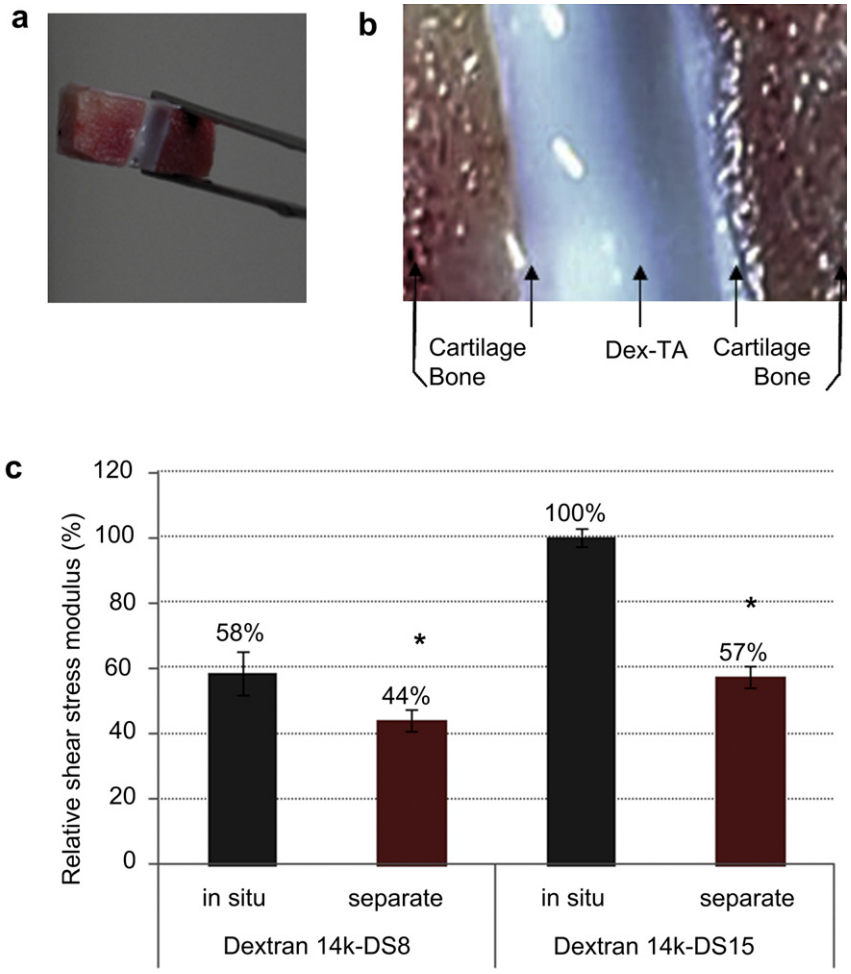

Fig. 1. Hydrogel adhesion and maximal shear modulus. a. and b. Construct consisting of two bovine explants of sub-chondral bone covered with a layer of cartilage of 1 cm^2 with a layer of *in situ* formed Dex-TA hydrogel between the explants. The final construct shows high stability when held horizontally between tweezers. c, Maximal shear modulus (relative) of cartilage with 14k-DS8 and 14k-DS15 Dex-TA hydrogels formed *in situ* and for cartilage combined with pre-gelated 14k-DS8 and 14k-DS15 Dex-TA hydrogels.

hydroxyphenyl groups present in both Dex-TA and cartilage matrix components, by evaluating whether Dex-TA based hydrogels promote self-attachment during the enzymatic crosslinking reaction. Additionally, we optimized the hydrogel features to enable cell invasion by incorporation of heparin components.

2. Methods

2.1. Synthesis of Dextran-Tyramine conjugates (Dex-TA)

Dextran from leoconostoc ssp. ($M_{n,dextran}$ = 14 k) and p-nitrophenyl chloroformate (PNC) were purchased from Fluka. Tyramine (TA), N,N-dimethylformamide (DMF) anhydrous 99,8%, hydrogen peroxide (H_2O_2), pyridine anhydrous 99,8%, lithium chloride (LiCl) and phosphorus pentoxide were obtained from Sigma-Aldrich. Heparin sodium (from porcine intestinal mucosa, molecular weight ranges from 3 to 30 kg/mol) was purchased from Celsus. Horseradish peroxidase (HRP, type VI, 256 purpurogallin unit/mg solid) was purchased from Sigma and used without further purification. Phosphate-buffered saline (PBS, 150 mM, pH 7.4) was purchased from B. Braun Co. LiCl was dried at 80 °C under vacuum in the presence of phosphorus pentoxide. Dex-TA and Hep-TA were synthesized as previously described [22–24]. The schematic representation of Dex-TA synthesis is shown in supplementary figure 1a.

2.2. Cell culture

Bovine primary chondrocytes were isolated from bovine knee articular cartilage by collagenase digestion and cultured in chondrocyte expansion medium (DMEM containing FBS, non essential amino acids, ascorbic acid, proline, penicilline/streptomycin and fungizone) as previously described [14]. Cells were incorporated into the hydrogels with a cell density of 10×10^6/mL (passage 0). The cell suspension was mixed with the polymer. The H_2O_2 and HRP solutions were added to the polymer/cell suspension and gelation occurred within 1 min. The schematic representation of Dex-TA crosslinking is shown in supplementary figure 1b. The amount of HRP used was fixed at 0.25 mg per mmol of TA moieties and the molar ratio of H_2O_2/TA was 0.20 (mol/mol). Constructs were cultured in chondrocyte expansion medium. The gelation reaction occurring in an artificially introduced bovine articular cartilage defect is depicted in Fig. 2a.

2.3. Articular cartilage in contact with Dex-TA: SEM and histological analysis

Dex-TA 14K-DS10 hydrogels with primary chondrocytes were prepared on top of bovine knee articular cartilage explants ($1 \times 1 \times 0.2$ cm). After 15 days of culture, the centres of the constructs were visualized using wet mode Scanning Electron Microscopy (SEM) (100% humidity, 10.0 kV, 3.7 Torr, XL 30 ESEM-FEG Philips). For high resolution SEM (HR-SEM), Dex-TA hydrogels were prepared on top of bovine knee articular cartilage squares ($0.5 \times 0.5 \times 0.2$ cm) and cross-sections of these squares, to evaluate whether the hydrogels are able to bind equally to the cartilage surface and to deeper cartilage zones. Biopsies from rat muscle and fat pad were also analysed. After fixation in 10% buffered formalin, the constructs were cut in half, dehydrated and lyophilized (CPD030 Balzers Critical Point Dryer). Dried constructs were then coated with gold (Cressington sputter coater) and sample centres were visualized using High Resolution (HR)-SEM. For histological analysis, hydrogel/cell constructs were formed on top of articular cartilage squares and collected after 21 days in culture. Samples of muscle and fat pad with Dex-TA were also processed for histology. Samples were embedded in paraffin and sections of 5 μm were used for the staining with Safranin O and with Picrosirius red. After rehydration, the samples were counterstained with haematoxylin and fast green. After rinsing with acetic acid solution (1%), the sections were stained with Safranin O solution (0.1%) and dehydrated. Slides were assembled with resinous medium for visualization using light microscopy (Nikon Eclipse E-400). Picrosirius red staining for total collagen visualization was performed according to the manufacturer's instructions (Polysciences) and analysed using a polarization filter.

2.4. Mechanical testing

Bovine articular cartilage explants were fixated in 4% buffered formalin and immobilized in the Dynamic Mechanical Analyzer (DMA) sample holders. Hydrogel samples of Dex-TA 14K-DS8 and 14K-DS15 were directly crosslinked onto the cartilage. As control, previously crosslinked hydrogels were placed onto the cartilage. The DMA 8000 (PerkinElmer) was calibrated in shear setup. The storage modulus and damping factor were measured at room temperature. Average and

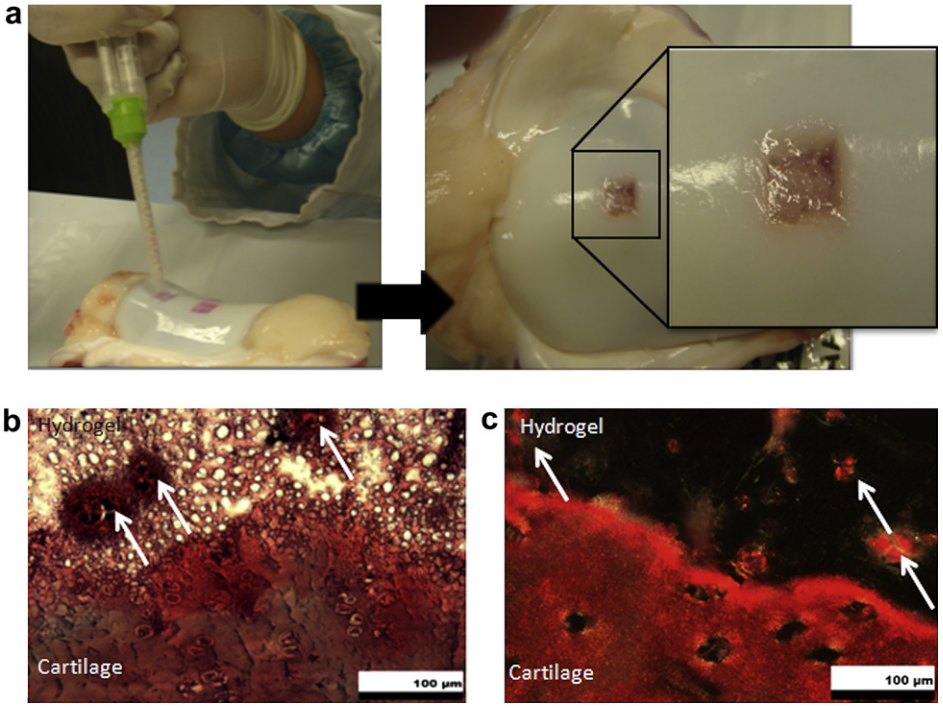

Fig. 2. Overview and histological evaluation of cartilage defects filled up with Dex-TA hydrogel. a. A cartilage defect in the bovine patella is filled up with a Dex-TA hydrogel combined with bovine chondrocytes. The gel is applied using a dual syringe in which one chamber contains the cell suspension (10 million cells per mL) and the polymer (final volume of 10% w/v) while the other chamber contains HRP and H_2O_2. After mixing both chambers' contents together along the circular final mixing compartment, gelation occurs within 1 min which results in total filling of the defect, as shown in the detailed picture of the simulated defect site at the right. b. Safranin O staining indicating in pink/red the accumulation of glycosaminoglycans. The white arrows indicate the cells incorporated in the hydrogel. A decreasing gradient of glycosaminoglycans (GAGs), from cartilage to the hydrogel, indicating that diffusion of GAGs through the interface takes place. In addition, safranin O staining is surrounding the chondrocytes embedded in the hydrogel, indicating newly formed GAGs. c. Picrosirius red staining, visualized by polarized light, demonstrates the presence of collagen fibrils at the cartilage/hydrogel interface and surrounding the chondrocytes embedded in the hydrogel, indicated by the white arrows. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

standard deviations were calculated over similar time frames. Values were afterwards converted into percentages, with Dex-TA 14k-DS15 *in situ* crosslinked onto cartilage set to 100%.

2.5. Micro-Raman spectroscopy

Fresh bovine articular cartilage pieces (1 × 3 × 3 mm) were collected. Dex-TA 14K-DS10, dissolved in PBS, was directly crosslinked on top of the cartilage. The gel and cartilage pieces without gel were used as controls. Raman measurements were carried out on non-fixed freshly assembled samples. Raman measurements were performed using a custom-built confocal Raman spectrometer. A Kr-ion laser (Coherent, Innova 90-K, Santa Clara, CA) with an emission wavelength of 647.1 nm was used as an excitation source. Raman spectra of a 30 × 30 μm scan area were collected (Power: 35 mW, 30×W objective, 50 ms/pixel accumulation time) as previously described [25,26]. A Raman calibration standard (toluene) with accurately known peak frequencies was used for wave number calibration of the spectra. All data manipulations were performed using routines written in MATLAB 7.4 (The Math Works Inc.). After hierarchical cluster analysis, a Raman cluster image and spectra for each cluster were obtained [25,27]. The peaks of interest were analysed using Origin software and Matlab.

2.6. Crosslinking of fluorescent labelled tyramide with components in the ECM of articular cartilage

Cy5-labelled tyramide (PerkinElmer) stock solution was prepared according to manufacturer's instructions and further dilutions were prepared in amplification diluent (PerkinElmer, USA). Articular cartilage explants (1 × 3 × 3 mm) were incubated for 1 h at 37 °C with Chondroitinase ABC (5146 units/mL in 0.01% BSA in PBS, Sigma). As a negative control, Chondroitinase ABC was replaced by PBS. Explants were washed with PBS and incubated with Cy5-labelled tyramide (1:50 or 1:100 dilutions of the stock solution, as supplied by the manufacturer), H_2O_2 and HRP for 10 min, under agitation. As a negative control, HRP was replaced by PBS. The volume ratio of HRP/H_2O_2 used was 286/218 and the volume ratio of (HRP + H_2O_2)/diluted tyramide was 1/4. Samples were visualized by fluorescence microscopy (Nikon E-400). All pictures were taken at the same exposure time. For quantification of fluorescence intensities, the mean colour of the pictures of the samples was determined using Corel Photo Paint 12 software. The colour scale ranges from 0 to 255, in which a value of 0 correlates with a pure black sample and a colour of 255 represents a pure red sample.

2.7. Cell adhesion and cell migration assay

Cell migration assays were performed using a transwell system. Dex-TA/Hep-TA hydrogels in ratios of 100/0, 75/25, 50/50 and 25/75 (final polymer content of 20% (w/v)) were placed on the bottom of migration plates (Kit CytoSelect 24-well Cell Migration Assay 8 μm, Colorimetric format; CBA-100 Cell Biolabs, Inc.). Chondrocytes were isolated from human articular cartilage, obtained after total joint replacement surgery. Cartilage explants were kept in culture for two weeks to allow isolation of chondrocyte progenitor cells, as described elsewhere [28], or immediately digested using collagenase type II (Worthington), to isolate the chondrocytes. Isolated cells were seeded in a density of 3000 cells per cm^2. Cells were expanded until use (passage 2). The insert was filled with 300 μL of a cell suspension containing 2 × 10^6 human chondrocytes (hChond) or chondrocyte progenitor cells (hCPC)/ml in serum-free medium and cultured for 24 h. Afterwards, the medium was carefully aspirated from the inside of the insert. The interior of the inserts was swabbed to remove all non-migratory cells. The inserts were transferred to a clean well containing 400 μL of Cell Stain Solution and were incubated for 10 min at room temperature. The stained inserts were washed several times and allowed to dry to air. Each insert was transferred to an empty well and 200 μL of Extraction Solution was added. After 10 min of incubation, each sample was transferred to a 96-well microtiter plate and the optical density (OD) at 560 nm was measured in a plate reader. The migration of cells within the hydrogels was also assessed. Dex-TA gels containing cells were prepared on top of Hep-TA gels without cells. Dex-TA gels contained 0.25 × 10^6 bovine primary chondrocytes (P2). Gels were removed from the wells and put upside down (Dex-TA on the bottom) in a cell culture plate, with chondrocyte expansion medium. Samples were fixated in 10% buffered formalin at day 1, 3, 7 and 14. Cryosections (20 μm thickness) were stained for 10 min with 0.1% toluidine blue solution (Fluka). After washing, samples were incubated with DAPI (100x diluted, Vector Laboratories) and analysed by light and fluorescent microscopy.

3. Results

3.1. Higher number of hydroxyphenyl groups enabled stronger adhesion to the host tissue

Adhesion of a biomaterial to native cartilage is considered as a critical step in integrative cartilage tissue repair [29]. Fig. 1a and b show a construct of bone-cartilage-hydrogel-cartilage-bone. Notably, Dex-TA with higher degree of substitution visually resembled cartilage and allowed the support of the whole construct. A dynamic mechanical analyzer (DMA) was used to measure the adhesion strength between cartilage and Dex-TA hydrogels. The shear setup was selected to determine the shear moduli of hydrogels with two different degrees of substitution: DS8 and DS15. In this way, the amount of hydroxyphenyl groups available for crosslinking within the polymeric network and onto the cartilage surface is almost doubled. The maximal shear moduli were determined both for hydrogels crosslinked *in situ* on top of the cartilage and apart from the cartilage (Fig. 1c). In the latter case the gel and cartilage were joined together before measurements. Interestingly, the shear moduli of Dex-TA hydrogels *in situ* formed on cartilage were higher than for hydrogels combined with cartilage after gelation. In addition, the maximal shear moduli for the gels based on DS15 were higher than those based on DS8. Thus, higher degrees of substitution lead to both mechanically stronger gels [22] and to higher adhesion strength between the hydrogel and the native tissue, which lead us to investigate further the

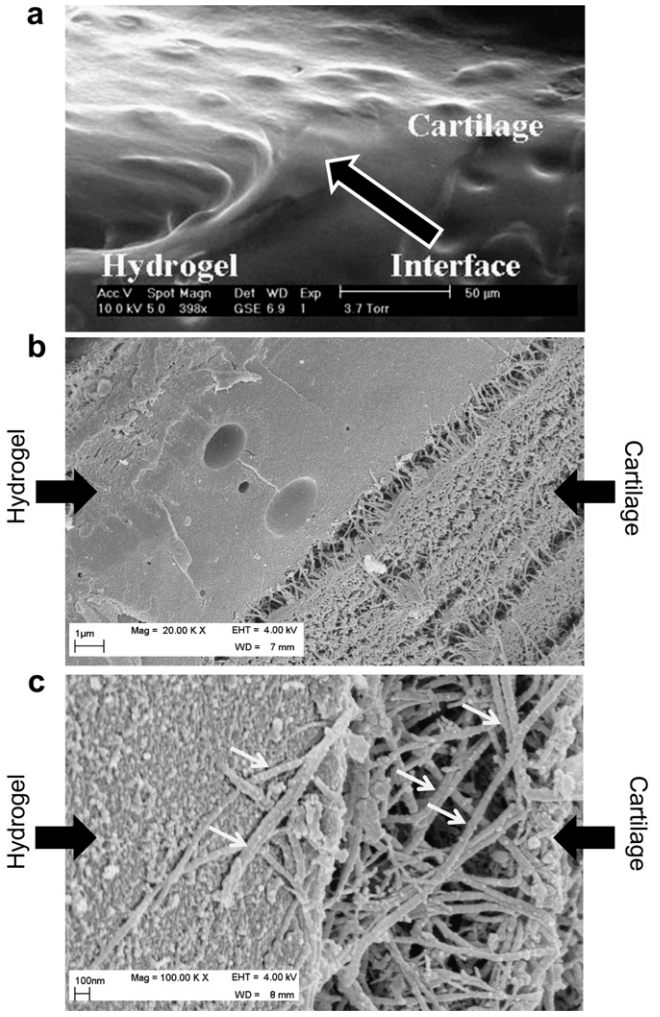

Fig. 3. Morphological analysis of the cartilage-Dex-TA hydrogel interface. a, Representative wet mode SEM images showing a smooth transition between Dex-TA hydrogels and cartilage. The arrow indicates the cartilage-Dex-TA hydrogel interface. c and d, Representative HR-SEM pictures showing increasing magnifications of the hydrogel-cartilage interface. The images are suggestive for a direct interaction between the hydrogel and collagen fibrils of which the D period is clearly visible at higher magnifications (with arrows). The magnifications are indicated in the figures.

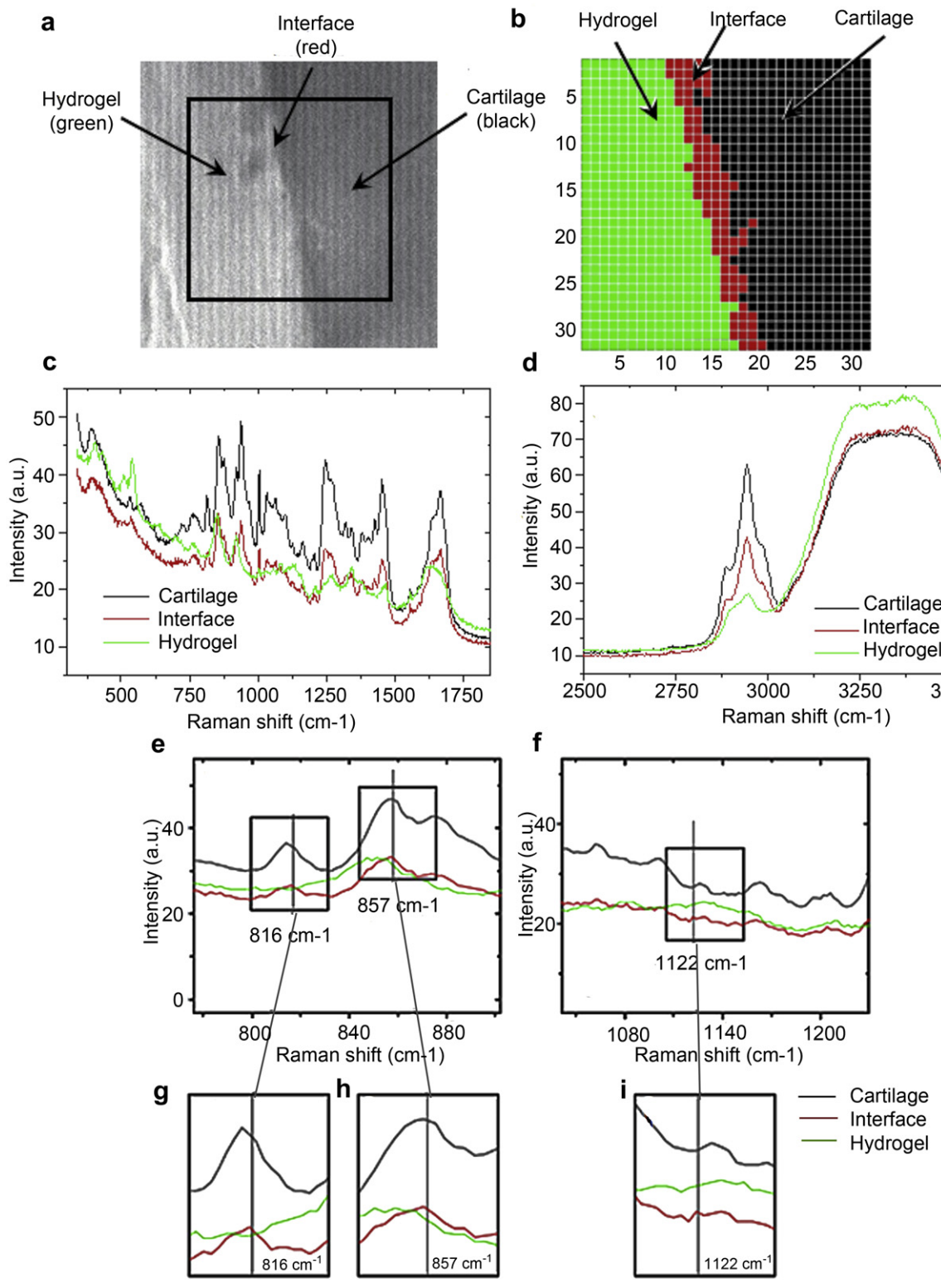

Fig. 4. Raman micro-spectroscopy of the interface area. a, White light micrograph showing the scanned interface region of 30 × 30 μm (boxed). In this area 1024 scans were taken. b, Hierarchical cluster analysis of 1024 Raman scans (Power: 35 mW, objective: 40×W, accumulation time 50 ms/pix) identifies 3 different spectra corresponding to the hydrogel only (green), the cartilage only (black) or the interface (red). c, d, Raman spectra of the Dex-TA/cartilage interface with: the cartilage region (black cluster), the interface region (red cluster) and the Dex-TA 14k-DS10 hydrogel region (green). c, The most informative region of the spectrum; d, Spectrum showing the water bands (Power: 35 mW, objective: 40×W, accumulation time 50 ms/pix). e-f, Spectral range selections of figure c, of $\Delta = 780–820\ cm^{-1}$ and of $\Delta = 1050–1230\ cm^{-1}$, respectively, showing peaks of interest. g, Selected area of e, showing a change at 816 cm^{-1}.h, Selected area of e, showing a change at 857 cm^{-1} i, Selected area of f, showing a change at 1122 cm^{-1}. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

hypothesis of covalent bonding of the gels to tyrosine residues of the cartilage.

3.2. Analysis of the hydrogel–cartilage interface showed evidence for gel-tissue integration

When simulating *in situ* gelation of Dex-TA polymers together with 10×10^6 chondrocytes/ml in an articular cartilage defect created in the bovine patella, we observed smooth filling of the defect (Fig. 2a). Consequently, we investigated the interface between the host tissue and the Dex-TA hydrogel in more detail. Representative histological analysis of sections showed the integration of Dex-TA hydrogels with articular cartilage, as shown in Fig. 2b and c. The constructs of gel-cells/cartilage were cultured for 21 days with no further addition of growth factors. A gradient of proteoglycans in the hydrogel was visible, as evidenced by the increase in intensity of Safranin O staining (glycosaminoglycans stain red/pink). This staining decreased with increasing distance to the cartilage interface (Fig 2b), suggesting integrative interaction between the hydrogel and the cartilage. Intense safranin O staining was also observed surrounding the chondrocytes. Picrosirius red staining, which is used to visualize collagens under polarized light, showed a highly intense staining in the interface area and in the pericellular matrix surrounding chondrocytes embedded in the hydrogel, as shown in Fig. 2c.

3.3. Bonding of the polymers to collagen fibrils in the cartilaginous matrix

To visualize the interface between *in situ* crosslinked Dex-TA hydrogels and articular cartilage at the micro and nanometre scale, two approaches were used: wet SEM and HR SEM imaging. In wet SEM, a smooth transition between Dex-TA hydrogel and articular cartilage was observed, as shown in Fig. 3a. Due to the high pressure, water was extracted from the hydrogel but not from the cartilage, chondrocytes in the cartilage are visible as spheres allowing easy discrimination of the gel and native cartilage. Secondly, HR-SEM was performed to analyse the interface at the nanometre scale. The cross-sections shown in Fig. 3b and c clearly showed attachment of the hydrogels to articular cartilage through interconnections with cartilage ECM components. It is likely that these ECM components are collagen fibrils, since the D period in the observed fibrils was clearly visible at high magnifications (Fig. 3c).

3.4. Formation of new C–C and C–O–C bonds suggestive of covalent bond formation

The intricate interaction of Dex-TA with collagen fibrils and the increase of adhesion strength when more hydroxyphenyl groups were present are strongly suggestive that the formation of covalent bonds with ECM proteins has taken place. We next used Raman micro-spectroscopy to identify the nature of the molecular interaction between the hydrogel and the cartilage (Fig. 4a). The interface of cartilage and the *in situ* formed Dex-TA was scanned and the spectra were analysed by hierarchal cluster analysis allowing discrimination of hydrogel only, cartilage only and interface spectra (Fig 4b–d). The red spectrum represents the average spectra of pixels corresponding to the interface region between hydrogel and cartilage. This interfacial region spectrum showed different spectral properties compared to the spectra of hydrogel and cartilage only. Detailed analysis showed the presence of spectral features of both cartilage and hydrogel in the interface (Fig. 4c and d). However, when enlarged also new features such as shifts in peaks, new peaks and reshaped peaks could clearly be identified as specific for this region (Fig. 4e–i). These new peaks are listed in Table 1, according to Movasaghi *et al.* [30]. These peaks could be assigned to collagens, polysaccharides and aminoacids (especially tyrosine). Interestingly, new peaks could also be assigned to remodelling of C–C and C–O–C bonds which are formed during the peroxidase mediated crosslinking. An intricate interaction between cartilaginous ECM proteins and the hydrogel was furthermore supported by the resulting interface spectra with unique characteristics after subtraction of the hydrogel only spectrum (supplementary figure 2).

Table 1
Spectral interpretations of the Raman spectra. The detected peaks of interest in the interface spectra, showing a shift when compared to the spectra obtained from cartilage and hydrogel, are attributed to a specific assignment, according to Movasaghi *et al* [30].

Detected peak (cm^{-1})	Assignment.
728	C–C stretching, proline (collagen assignment)
816	815:Proline, hydroxyproline,Tyrosine, U_2PO_2 stretch of nucleic acids 817:C–C stretching (collagen assignment)
857	855-856:Tyrosine C–C stretching 856:C-C vibration of the collagen backbone 859:Tyrosine, collagen
1102	1100:C-C vibration mode of the gauche-bonded chain
1122	U_s(C-C) skeletal,U_{sym}(C–O–C)(polysaccharides,cellulose)
1332	–C stretch of phenyl (1) and C_3–C_3 stretch and C_5–O_5 stretch CH_α in-plane bend

3.5. HRP enabled covalent crosslinking of tyramide residues to cartilage matrix

The enzymatic reaction occurring during crosslinking represented in Fig. 5c on cartilaginous tissue was mimicked by fluorescent labelled tyramide to confirm whether the tyramine residues in Dex-TA were indeed involved in the adhesion reaction. Fluorescent labelled tyramides (TyrCy5) were used to visualize tyramides retained on the cartilage surface after the peroxidase mediated crosslinking resulting in fluorescence, represented in Fig. 5a. Unbound tyramides were washed off and did not result in fluorescence of cartilage explants. The effects of pre-digestion of articular cartilage by chondroitinase and various concentrations of the labelled tyramide in combination with H_2O_2 and HRP were addressed. Fig. 5b shows a clear difference in the fluorescence level between the samples incubated with and without HRP. When no HRP was present the fluorescence level was lower. Moreover, a decrease in the fluorescence level could be detected when using 1:100 instead of 1:50 diluted tyramides.

3.6. Covalent bonding of hydrogels to collagen-rich tissues other than cartilage

We next examined whether *in situ* formed Dex-TA hydrogels could also covalently bind to other tissues than cartilage by evaluating their interaction between the hydrogels and muscle or fat pad. The ECM of these two tissues consists mainly of collagen type I with variations in quantity and structural organization. HR-SEM and histological evaluation was performed on constructs of tissue explants with an *in situ* formed layer of Dex-TA hydrogel. HR-SEM of the muscle–hydrogel interface showed interactions of the hydrogel with collagen fibrils (Fig. 6a). The fat pad–hydrogel interface observed by HR-SEM provided also evidence for an interaction between the hydrogel and collagen fibrils but in line with the lower quantity of collagen fibrils in fat tissue, the interactions were less abundant (Fig. 6b). Histological analysis showed

a smooth interface area without any gaps between muscle and Dex-TA hydrogels resembling the interface between cartilage and hydrogels (Fig. 6a, right image showing a section stained with H&E). The interface morphology between the fat pad and Dex-TA showed areas with gaps (Fig. 6b, right image showing a section stained with H&E), suggesting that the bonding of the *in situ* formed hydrogel to different tissues is dependent on the amount of collagen in the extracellular matrix of the respective tissue.

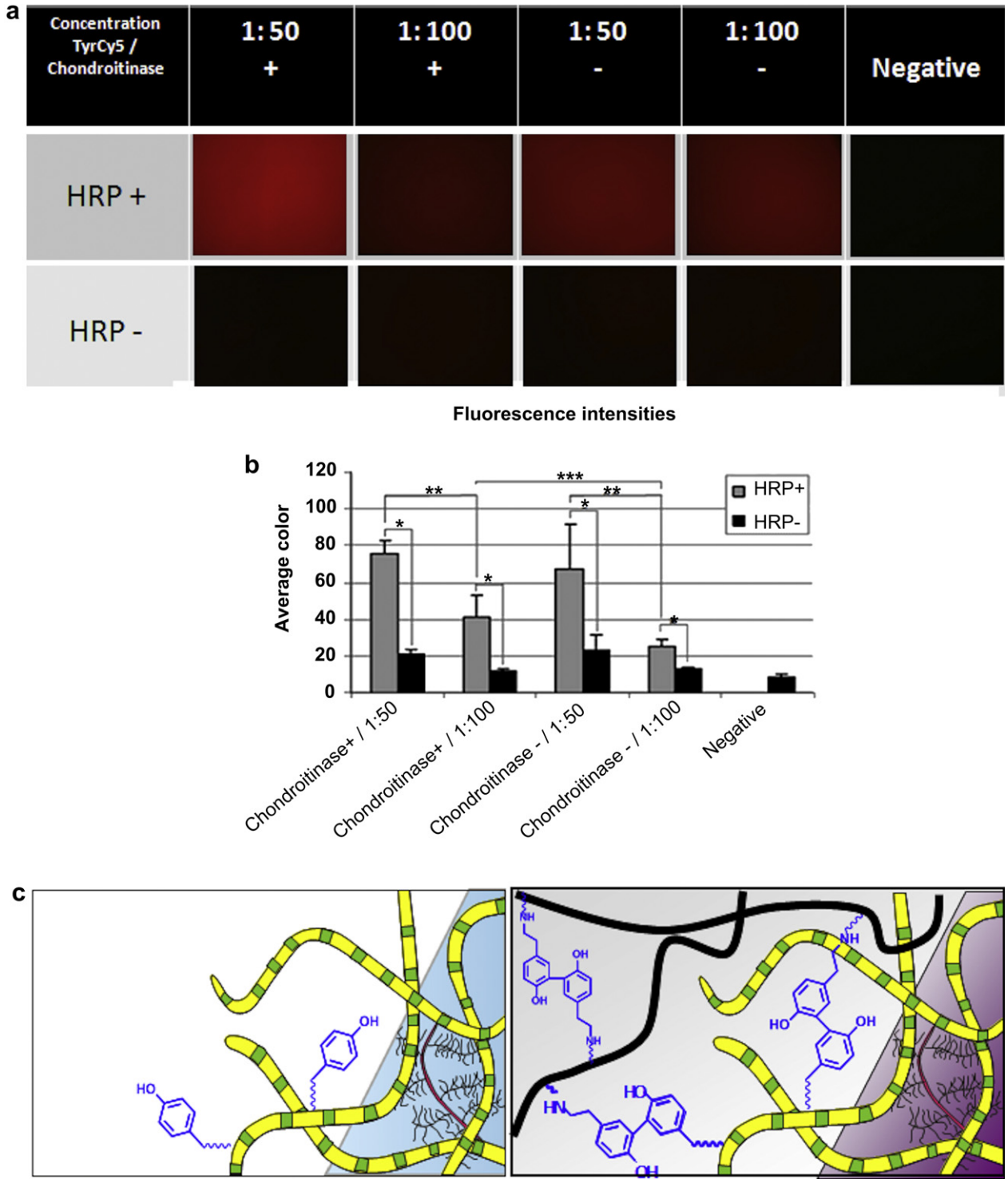

Fig. 5. Enzymatic crosslinking of Cy5-labelled tyramide on bovine articular cartilage. a, Fluorescent microscopy images show representatives ($n = 4$) of bovine articular cartilage incubated with or without chondroitinase, with and without HRP, with H_2O_2 and different concentrations of TyrCy5 (diluted 1:50 or 1:100). b, Fluorescence quantification of bovine articular cartilage incubated with and without chondroitinase, in the presence or absence of HRP and H_2O_2 with different concentrations of TyrCy5 (diluted 1:50 or 1:100). The mean colour of the pictures was determined for quantification of fluorescence intensities (average of 3 spots within each sample, $^*P < 0.05$), average colour: 0 = black, 255 = red. c, Schematic summary of the postulated enzymatic crosslinking between Dex-TA conjugates and collagen molecules present in the cartilage ECM via tyramine-tyrosine covalent bond formation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

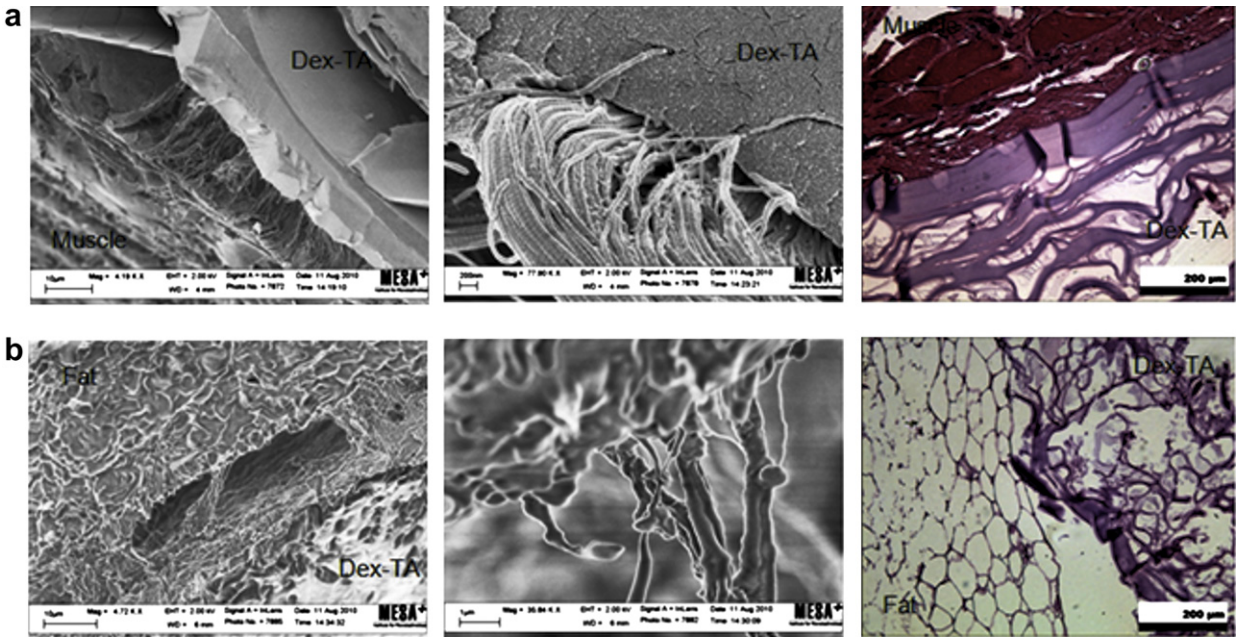

Fig. 6. HR-SEM and histological images, showing the interface between other collagen-rich tissues and Dex-TA hydrogel. a, HR-SEM pictures showing the attachment of the hydrogels to muscle tissue via bonding to collagen fibrils (left and middle) and a representative histological section with H&E staining demonstrating the smooth transition without gaps at the muscle/hydrogel interface (right). b, HR-SEM pictures showing limited attachment of the hydrogels to fat tissue via bonding to collagen fibrils (left and middle) and a representative histological section with H&E staining, demonstrating the irregularities and poor attachment at the fat/hydrogel interface (right).

3.7. Incorporation of Hep-TA enabled cell adhesion and triggered cell homing in Dex-TA hydrogels

In addition to the characterization of the self-attachment properties of Dex-TA, the potential of this hydrogel to be used as a cell-free system was evaluated. Since the cell interaction potential of Dex-TA is limited, hydrogels were prepared with Dex-TA and Hep-TA hydrogels in different ratios to evaluate whether this strategy could induce cell recruitment. The cell migration potential of these hydrogels was determined by a trans-membrane migration assay. Two different cell types were used; human chondrocytes and chondrocyte progenitor cells, which are known to exhibit a higher migratory potential [28]. The results show that increasing contents of heparin induced higher cell homing towards the hydrogels, with no significant difference between the two cell types (Fig. 7a). In a dynamic cell seeding experiment, cells attached to the outer surface of a Dex-TA hydrogel were observed but these cells invaded the hydrogel only to a limited extent (Fig. 7b). To test whether the Dex-TA hydrogels were compatible with cell migration Dex-TA hydrogels were incorporated with chondrocytes. Subsequently, a cell-free Hep-TA hydrogel was prepared on top of these cell loaded Dex-TA hydrogels (Fig. 7c). Migration of cells through the Dex-TA into Hep-TA gels could be tracked as the two gel types could be distinguished by toluidine blue staining which stained heparin but not dextran. Interestingly, cell migration against gravity into Hep-TA hydrogels was observed indicating that Dex-TA hydrogels are compatible with cell migration and that the incorporation of Hep-TA renders hydrogels chemoattractant properties (Fig. 7d), likely due to the high affinity of heparin to bind with growth factors and chemokines.

4. Discussion

Hyaline cartilage regeneration remains challenging. Tissue engineering strategies may provide promising avenues for restoring function when the tissue's self-renewal capacity cannot overcome the degeneration caused by disease, severe injury or age-related wear. Polymeric scaffolds that can be applied in minimally invasive procedures are of particular interest. These scaffolds can be designed to orchestrate ingrowth of tissue progenitor cells and facilitate the repair process. In situ gelating hydrogels of natural or synthetic polymers provide an excellent alignment with surrounding tissues, and both cells and bioactive molecules can easily and homogeneously be incorporated [31]. However, despite all the advantages of such hydrogels, adequate integration of the hydrogel with native tissue, which is a prerequisite for long term integration of neo-tissue, is still an unresolved challenge for most systems [32,33].

Previously, we and others have described hydrogels of natural polymers that gelate *in situ* by an enzymatic crosslinking reaction [14,15,22–24,34]. Particularly Dex-TA and Hep-TA hydrogels have shown promising features for application in cartilage tissue engineering. These biodegradable hydrogels are formed by an enzymatic crosslinking reaction mediated by HRP The crosslinking reaction occurs under mild conditions and within one to 2 min and is compatible with incorporation of cells [22–24]. The integration and interaction between these hydrogels and native cartilage is of major importance, and, therefore, the main focus of this study.

Initial adhesion is the key to fast formation of homogeneous tissue with sufficient mechanical stability and functionality [35]. Considering that the formation of di-tyramine, di-tyrosine and the oxidative coupling of other phenols occurs under the influence of HRP and H_2O_2 [36], we hypothesized that tyramine-tyrosine bonds could be formed during the *in situ* crosslinking of tyramine-containing hydrogels onto tyrosine-containing cartilaginous ECM proteins (Fig. 5c). A comparable mechanism has been described for poly(ethylene glycol)diacrylate (PEGDA) crosslinking on cartilage via tyrosyl radicals [35]. However, this hydrogel-cartilage crosslinking reaction is not based on phenol–phenol coupling and it demands a multi-step approach, involving aggressive oxidation methods with UV exposure and high H_2O_2 concentration (5%). In contrast, mild oxidation methods with low H_2O_2 concentrations (less than 0.1%) are used in the formation of Dex-TA hydrogels.

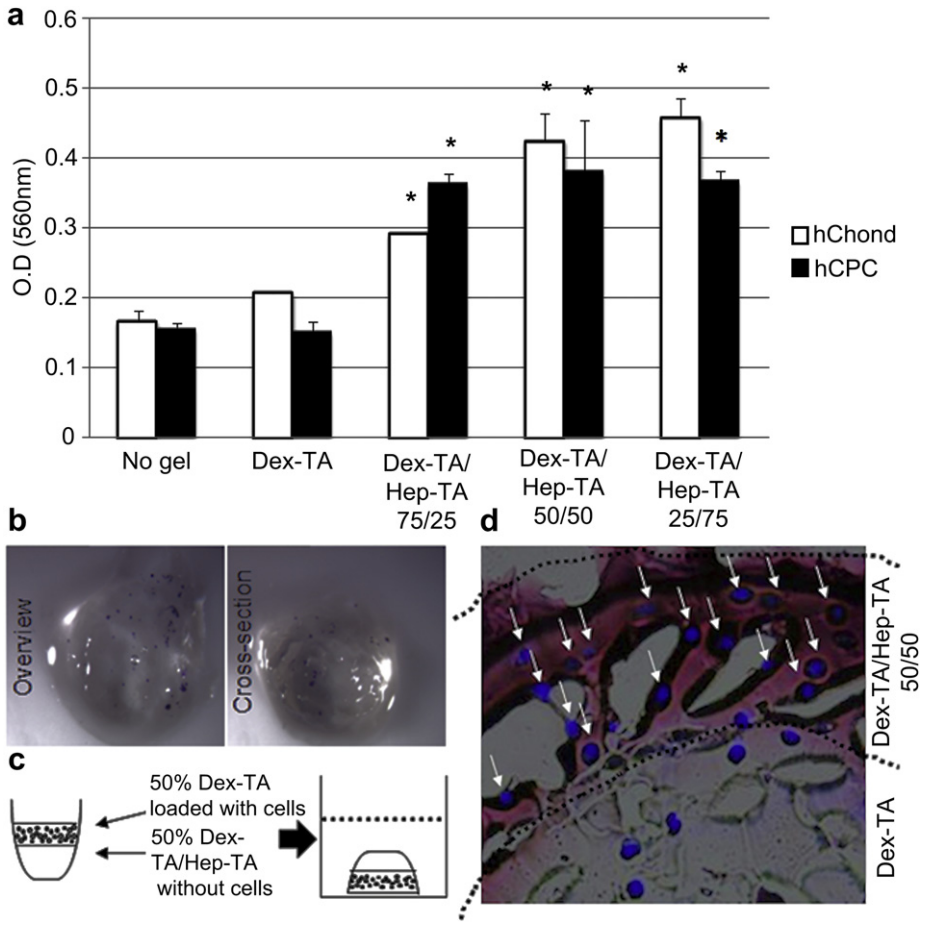

Fig. 7. Evaluation of the cell migratory potential of Dex-TA-based hydrogels. a. Cell migration assay using human chondrocytes (hChond) and human chondrocyte progenitor cells (hCPCs). Dex-TA was mixed with Hep-TA in different ratios: 100/0, 75/25, 50/50 and 25/75 to prepare hydrogels and the migratory cells were quantified. Increasing contents of heparin induced higher cell homing towards the hydrogels, with no significant difference between cell types. b. Cell adhesion onto Dex-TA hydrogels was observed, after dynamic cell seeding. The hydrogels were kept in a spinner flask for 7 days and afterwards an MTT assay was performed. The metabolically active cells are stained in purple and can be visualized both at the surface and, to a lesser extent, within the hydrogel, as shown by the cross-section. c. Representation of the assembly of constructs of Dex-TA hydrogels containing cells, with Dex-TA/Hep-TA 50/50 gels, without cells, on top. d. Migration of cells through the Dex-TA into Dex-TA/Hep-TA 50/50 gels. Toluidine blue staining was used to stain the heparin fraction. Cells were stained with DAPI, which marks in blue the cell nuclei. Cell migration against gravity towards Dex-TA mixed with Hep-TA hydrogels was detected after 24 h in culture, as cells were observed in the initial cell-free Dex-TA/Hep-TA 50/50 fraction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Here, we report the mechanism of adhesion between cartilage and peroxidase crosslinkable Dex-TA hydrogel. Mechanical analysis of the hydrogel using a rheometer has been previously described, providing information about the storage moduli of Dex-TA with various degrees of substitution and molecular weight [22]. Interestingly, our data showed that higher degrees of substitution do not only lead to stronger gels, but also to higher adhesion strength between the hydrogel and the native host tissue. This means that a higher number of tyramine groups are available in the polymer with higher degree of substitution, not only favoring the enzymatic crosslinking reaction between Dex-TA conjugates, but also between the conjugates and cartilage.

From the morphological analysis of the interfacial area between the host tissue and the hydrogel, we concluded that Dex-TA hydrogels did not only attached to the articular cartilage surface, but also to deeper zones of the cartilage, which can be a useful property in the treatment of full thickness or sub-chondral cartilage defects. Furthermore, three interesting phenomena were identified in the HR-SEM characterizations: first, a zone of approximately 2 μm wide was observed where the collagen fibrils in the articular cartilage could be identified; second, collagen fibrils were integrated into the hydrogel and finally, the collagen fibrils at the interface clearly reflected alignment towards the hydrogel. We addressed whether these adhesion events also take place when using other collagen-rich tissues. Similar results were obtained for muscle tissue but not for fat pad. These differences could be explained by the differences in collagen types, availability of collagen and, thus, by the availability of tyrosine motifs in these tissues. Taken together, these observations strongly indicated the involvement of collagen fibrils in the immobilization of Dex-TA hydrogels onto articular cartilage and other collagen-rich tissues, during *in situ* gelation.

Covalent bond formation between PEGDA and cartilage analysed by ATR-FTIR has been previously reported [35]. However, Raman spectroscopy performs better than ATR-FTIR in aqueous environments, therefore this technique is more adequate for the study of biological tissues and hydrogels [37,38]. This spectroscopic method is non-invasive, thus, does not demand any processing of the samples prior to use. Raman spectroscopy also allows for the identification of functional groups, bonding types and molecular conformations [30]. Previously, in our group, we have described the molecular finger-print of both dextran and cartilage tissue alone, using Raman Spectroscopy [27,39]. In the present study, we described the unique molecular finger-print of the interfacial area

between the Dex-TA hydrogel and cartilage. Raman microspectroscopy analysis indicated the involvement and reorganization of collagen, polysaccharides and amino acids, especially tyrosine, at the hydrogel/cartilage interface. Furthermore, and of great interest, is the fact that the cluster identified as being the interface between hydrogel and cartilage, when represented in a Raman cluster image, corresponded to a zone of 2–3 μm wide, comparable to the width of the region in which reorganized collagen fibrils were identified in the HR-SEM characterization.

The enzymatic reaction occurring during crosslinking on cartilage was mimicked by fluorescent labelled tyramide, to confirm whether the tyramine residues in Dex-TA were involved in the adhesion reaction. Labelled tyramides were found to be crosslinked on articular cartilage surfaces. The reaction was more efficient when more tyramide groups are present. Moreover, pre-treatment with chondroitinase, which degrades polysaccharides in cartilaginous ECM [40], induced higher fluorescence intensity. This increase occurred because collagens were likely more accessible for the tyramides, HRP and H_2O_2. Indeed the presence of HRP appeared to be essential to crosslink tyramine residues to cartilage matrix proteins. This mechanism is illustrated in supplementary figure 1c. However, we cannot exclude that the obtained results could also be due to large tyramide complexes that were trapped in the extracellular matrix or due to deeper penetration of tyramides as a result of the digestion treatment.

Finally, an enhanced cell migratory effect in Dex-TA hydrogels was achieved by the incorporation of Hep-TA conjugates. Cells were likely attracted by the heparin, which might have performed as a driving force for migration of cells through the Dex-TA gel [41]. Heparin is a glycosaminoglycan, widely used as anticoagulant and antilipemic agent [42], which interacts with proteins involved in cell adhesion, migration, proliferation and differentiation [41,43]. Cell migration against gravity in Dex-TA hydrogels was possible and Hep-TA hydrogels were indeed able to induce cell recruitment. Attracting cells passively to the biomaterial/articular cartilage interface may lead to improved tissue integration. Moreover, because cells can be attracted from surrounding tissues, combinations of Hep-TA and Dex-TA may be used as a very effective cell-free system.

5. Conclusion

We show here that it is feasible to fill cartilage defects with an *in situ* gelating and mechanically stable hydrogel of natural polymers. During the crosslinking reaction, Dex-TA hydrogels firmly attached to the cartilage ECM by covalent bonding to tyrosine residues in collagen fibrils. In addition, by mixing combinations of natural polymers, for instance Dex-TA with Hep-TA, these hydrogels can be tailored to attract and facilitate cellular ingrowth of chondrocytes or chondrocyte progenitor cells. Thus, hydrogels containing mixtures of Dex-TA and Hep-TA conjugates are promising biomaterials for the development of a cell-free system that can repair tissue defects, not only in cartilage but also in other collagen-rich tissues.

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.biomaterials.2012.01.001.

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