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Cartilage-targeting dexamethasone prodrugs increase the efficacy of dexamethasone

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Abstract

Intra-articular administration of glucocorticoids such as dexamethasone is a common treatment for osteoarthritic inflammation and pain. Despite its potent anti-inflammatory properties, multiple barriers hinder the drug's effectiveness in the articular space. In particular, the high turnover rate of the synovial fluid and the dense cartilage extracellular matrix (ECM) lead to poor drug penetration into cartilage. In order to increase the infiltration and retention time, two dexamethasone prodrugs were developed. Firstly, dexamethasone was conjugated to polycationic chitosan, which led to deep and sustained infiltration of the drug into full thickness cartilage, due to its strong electrostatic interactions with the high negative fixed charge of the cartilage ECM. Secondly, dexamethasone was conjugated to a collagen type II-binding peptide, WYRGRL, and this prodrug was shown to be retained in the deep zones of cartilage through specific interactions with cartilage-specific collagen type II bundles. In both cases, active dexamethasone was released from the carrier by ester linkage hydrolysis. Complexing dexamethasone with either chitosan or collagen type II-affinity carriers increased its binding and therapeutic efficacy inside cartilage, compared to free drug. Both dexamethasone conjugates significantly reduced levels of inflammatory markers and slowed the loss of glycosaminoglycans in an *ex vivo* model. A single dose of a cartilage-targeting dexamethasone prodrug represents a promising alternative to the repetitive glucocorticoid injections needed to compensate for its rapid clearance from the joint cavity.

1 Introduction

Osteoarthritis (OA) has been defined by Loeser et al. as "an abnormal remodeling of joint tissues driven by a host of inflammatory mediators"[1], leading to pain and loss of joint function.[1] This complex disease affecting the whole joint impairs a significant proportion of the population and leads to serious disabilities. There are two major types of osteoarthritis. The development of primary OA is associated with aging and obesity as main risk factors and generally affects adults above 60 years old.[2] Among younger adults, secondary OA frequently occurs post-traumatically, following a joint injury. Shortly after trauma, the gradual death of the resident cells induces joint inflammation and starts the catabolic cascade of OA.[3] The low-grade chronic inflammation and accompanying pain, joint stiffness and swelling, have an impact on joint function and cause reduced mobility. In osteoarthritic joints, fibrillation is observed, and significant glycosaminoglycan (GAG) loss occurs, leading to the destruction of the joint surface.[4,5] Matrix degradation and cell death induce surviving chondrocytes, synoviocytes, synovial fibroblasts and synovial macrophages[6] to increase the secretion of major inflammatory cytokines (IL-1, IL-6, IL-8, TNF- α , interferon- γ , COX-2, NOS-2). It is believed that continuous exposure to these inflammatory signals leads to the intensification of debilitating OA symptoms. The cartilage tissue is irreversibly deteriorated, leading to permanent loss of load-bearing cartilage function.[3,7] In end-stage OA, partial or total joint replacement surgeries are generally unavoidable to reduce pain and disability.[2] Considering the increased aging of our society, the number of these surgeries and indirect costs engendered by OA are expected to increase dramatically.

Fortunately, some solutions exist to alleviate OA symptoms and slow down the inflammatory cascade. A common therapy is the administration of glucocorticoids, orally or via intra-articular injections. This class of steroid hormones exhibits strong anti-inflammatory and chondroprotective properties.[8] It was shown to induce the inhibition of matrix metalloproteinase (MMP)[9], prostaglandins[10], pro-inflammatory cytokines[11] as well as to prevent cell death[12] and glycosaminoglycan loss.[3] There are five corticosteroids clinically approved for intra-articular injections.[13] Amongst them, dexamethasone is a synthetic corticosteroid known to have a potent and long-lasting anti-inflammatory effect. However, these benefits are strongly hindered by a low intra-cartilage diffusion rate and a quick clearance from the joint space. Short-term systemic administration glucocorticoids was demonstrated to be safe and well tolerated.[14] Clinicians sometimes prescribe dexamethasone

(by intravenous injection) to reduce postoperative pain and nausea after total joint arthroplasty[14,15], but they are reluctant to its long-term use because of reported off-target effects to skin, eyes, cardiovascular and gastrointestinal systems, bones, muscles or central nervous system, which were shown to be proportional to the duration and dosage of the treatment.[14,16]

The bioavailability of a drug is usually limited by trans-capillary diffusion, which becomes infinitesimal if the tissue is avascular, such as cartilage. Local administration is largely preferred to systemic delivery in order to reduce side effects and increase bioavailability.[17–19] Additionally, the amount of drug administered is reduced to a precise and effective drug dosage, allows the delivery of molecules that would otherwise cause serious body harm (via systemic delivery), and avoids fast renal clearance. However, despite these multiple advantages, it is difficult to circumvent fast lymphatic drainage from the joint cavity.[20] While a high drug concentration can be temporarily achieved within the synovial fluid, the diffusion rate through cartilage remains low in comparison to the clearance rate, leading to subtherapeutic intra-cartilage concentrations. More precisely, the intra-articular half-lives for corticosteroids have rarely been found to exceed 12 hours.[21,22] The efficacy of therapeutics is dependent on their longevity within the joint space and on the infiltration through the tissues.

Two common strategies to slow down drug clearance from the joint are to either lower drug solubility (e.g., by keeping it longer in crystalline form[20]) or to specifically target the drug to cartilage tissue.[23] To achieve the latter, different cartilage-targeting vehicles and loading strategies can be used. Longer release times, without burst release, can often be achieved through covalent conjugation of a drug to a carrier as opposed to physical entrapment in nano- or microparticles.[24,25] The formation of a drug-carrier conjugate, or *prodrug*[26], involves the reversible linking of a carrier molecule, or promoiety, to the active drug. The carrier can be useful to slow elimination from the body, improve solubility or targeting specificity.[26] For example, in order to increase the lipid solubility of dexamethasone and allow its liposomal delivery, dexamethasone palmitate was developed, consisting of a long aliphatic chain attached to the drug via an ester bond.[27] It could be shown that the addition of the hydrophobic tail induced a 5.6x increase in anti-inflammatory activity.[27] This form of dexamethasone, like all modified drugs, required new regulatory approval[28] and is currently available in China and Japan (Limethason®, Mitsubishi Pharmaceuticals; Lipotalon®, Merckle).

This study aims at specifically delivering dexamethasone (Dex) to deep zones of cartilage tissue, which is important since the majority of chondrocytes secreting the pro-inflammatory cytokines are located within the middle and deep zones of cartilage. Cartilage tissue is largely composed of a dense network of negatively-charged glycosaminoglycans (GAGs) and collagen type II fibrils. Both components can be used to target a drug safely and specifically to deep regions of cartilage tissue (**Fig. 1**). In the first approach, the high negative fixed charge density in the cartilage ECM uses electrostatic interactions to target positively-charged drug carriers. The presence of fixed GAGs throughout the cartilage thickness not only attracts the carriers but considerably extends their residency time, leading to a higher cellular uptake.[23] For example, Avidin (a positively charged protein) was conjugated to Dex and showed increased efficacy *in vitro*. [23,29] Improved infiltration, suppression of injury-induced joint swelling and catabolic gene expression could be shown when injecting Avidin-dex intra-articularly in a post-traumatic OA rabbit model.[30] Although providing strong binding, electrostatic interactions are rather unspecific and could lead to off-target delivery. In parallel, we focused on developing a more specific approach by using a collagen type II-binding peptide. In particular, the hexapeptide WYRGRL was shown to successfully deliver peptide-functionalized nanoparticles to cartilage by targeting collagen type II.[31] A clinical application of this peptide was its conjugation to MRI contrast agents and visualization of osteoarthritic lesions.[32] In the present study, dexamethasone was conjugated to the promoieties via a hydrolysable carboxylic acid ester linker. After synthesis, the prodrugs were characterized, and enhanced retention and efficacy were demonstrated. Taken together, we believe

that these prodrugs represent an attractive alternative to intra-articular injection of dexamethasone by targeting cartilage tissue effectively.

Fig. 1 (two-column) Two strategies were developed to increase dexamethasone efficacy in osteoarthritic cartilage. Dexamethasone prodrugs were formed by conjugating the drug to chitosan or a collagen type II-binding peptide. In the first case, the drug-polymer complex binds to the negatively-charged ECM by electrostatic interactions while in the second case, specific interactions between collagen fibrils and the peptide WYRGR retain the prodrug. Hydrolysis slowly takes place, releasing the active drug from its carrier, thus decreasing joint inflammation and GAG loss.

2 Materials and methods

2.1 Chemicals

Unless otherwise stated, solvents and reagents were purchased from Sigma-Aldrich, Buchs, Switzerland.

2.2 Drug release study

The electrospinning of Dex-loaded fibers was performed as previously described.[33] Briefly, poly(ϵ -caprolactone) (PCL, 80 kDa, 440744) was dissolved at 13% w/v in a chloroform/ethanol 4:1 mixture and electrospun on a commercial electrospinning apparatus (EC-CLI, IME Technologies, Netherlands). The physically-loaded fibers (■) were prepared by adding dexamethasone at 0.5 mg/ml to the electrospinning solution. To prepare the covalently-immobilized Dex-loaded fibers (▲), lower molecular weight PCL was first synthesized by ring opening polymerization. 5 g ϵ -caprolactone (17344, Acros Organics, Geel, Belgium) was dissolved in 50 ml anhydrous tetrahydrofuran (THF) and heated to 80°C. 280 μ l of stannous 2-ethylhexanoate ($\text{Sn}(\text{Oct})_2$) and 16 μ l of H_2O (leading to a carboxylic acid terminus) were added as catalyst and initiator, respectively. The solution was heated under reflux for 72h. The polymerized ϵ -caprolactone was precipitated in 400 ml diethyl ether and dried under vacuo. To conjugate dexamethasone, 500 mg of the previously synthesized PCL was dissolved in 10 ml anhydrous dichloromethane (DCM). To this solution, were added 4-(Dimethylamino)pyridine (DMAP, 31 mg, dissolved in 300 μ l DMF), N,N' -Diisopropylcarbodiimide (DIC, 39 μ l) and dexamethasone (Dex, 59 mg, Acros Organics, Geel, Belgium, dissolved in 300 μ l DMF). After 24 hours, the solution was precipitated in cold diethyl ether/methanol 9:1. The slurry was centrifugated, the precipitate resuspended in chloroform, precipitated again in cold diethyl ether, filtrated and dried. For electrospinning, PCL-Dex was added to the high molecular weight PCL solution at 4% w/v. The peptide WYRGRGE-dex (◆) was prepared as described in the section "Peptide synthesis". To account for enzymatic hydrolysis, we added 0.1 or 10 U/ml of carboxylesterase (E3019, Sigma-Aldrich, Buchs, Switzerland) to selected WYRGRGE-dex samples (◇).

The release profiles of chemically cross-linked dexamethasone vs physically-loaded dexamethasone were determined in PBS + 0.1% BSA, pH 7.4, at room temperature, with shaking. From the electrospun PCL membranes, $\varnothing$ 6 mm punches were cut and immersed in 1 ml buffer, in a sealed container. The peptide WYRGRGE-dex was dissolved at 100 μ M in the release buffer. At each time point, a small aliquot was taken and quantified by HPLC, using the following parameters: column: ACE Equivalence C18, 4.6 $\times$ 250 mm, 5 μ m (Advanced Chromatography Technologies, Aberdeen, United Kingdom) kept at 35°C; eluent: ACN/MeOH/ KH_2PO_4 (10 mM, pH 6.8); detector: 240 nm; retention time (Dex): 6.9 min; run time: 12 min. The data were fitted using a first-order kinetics model (equation (1)) and half-

life time corresponding to was extracted (equation (2)). The added volumes used for the quantification was $\leq 10\%$ of the total volume.

$$Y = Y_0 - Y_0 * e^{-kx} \quad (1)$$

$$t_{1/2} = \frac{\ln(2)}{k} \quad (2)$$

2.3 Dexamethasone functionalization

The following reaction was performed under argon atmosphere. Dexamethasone (50 mg, 0.127 mmol, 1 eq, Acros Organics, Geel, Belgium) and methacryloyl chloride (37 μ l, 0.381 mmol, 3 eq) were dissolved in 1 ml dry DMF. *N,N*-Diethylmethylamine (2 $\times$ 50 μ l) was diluted in a small amount of dry DMF and added slowly to the reaction mixture. The solution turned yellow, and formation of smoke was observed. The reaction was allowed to proceed under stirring for 48 hours at RT. The crude mixture was purified by HPLC; column: Agilent Prep 100 Å C18, 10 μ m, 21.2 $\times$ 250 mm cartridge (PrepHT) at isocratic conditions in ACN/MeOH/KH₂PO₄ (10 mM, pH 6.8) 30:26:44. Dexamethasone methacrylate eluted at 5.4 min while unmodified dexamethasone eluted at 8.8 min. The purified compound was then lyophilized to yield a light yellow solid. ¹H (MeOD) δ (ppm), methacrylic protons: 1.99 (s, 3H, –CH₃), 5.71 (s, 1H, =CH₂), 6.19 (s, 1H, =CH₂).

2.4 Chitosan conjugation

A 1% chitosan (C3646, Sigma-Aldrich, Buchs, Switzerland) solution was prepared by overnight dissolution in 1% acetic acid. The chitosan used had an effective deacetylation degree of 88% and based on the viscosity, the molecular weight range was estimated to be 100-200 kDa (not specified by the manufacturer). To 800 μ l of this solution, were added dexamethasone methacrylate (100 μ l, 40 mM in DMSO) and cat. FeCl₃ (100 μ l of 0.1 M in H₂O, filtered).[34] The reaction was performed 48 hours at RT, under gentle orbital shaking. The polymer was analyzed by size exclusion chromatography to confirm the successful conjugation of chitosan. Column: Agilent AdvanceBio SEC 300Å, 2.7 μ m; eluent: piperazine 100 mM, pH 5.0; temperature: 30°C; flow rate: 1.0 ml/min; runtime: 18 min; detector: 214 nm (chitosan backbone), 240 nm (dexamethasone). Note: the conjugation protocol was first established with a fluorescent analog (umbelliferone).

Surface ζ potentials were analyzed by a zetasizer at 50 V (Zetasizer Nano ZSP, Malvern Panalytical Ltd., Royston, UK). The polymer concentration was kept at 1 mg/ml and the measurement was performed at pH 5.0 and pH 7.4.

2.5 Peptide synthesis

The collagen type II binding peptides were based on the WYRGRL sequence.[31] For the conjugation of dexamethasone, an orthogonal glutamic acid residue (E, Fmoc-Glu(O-2-PhiPr)-OH (Merck Millipore, Darmstadt, Germany)) was added at the C-terminus to allow reversible conjugation to Dex by Steglich esterification, leading to the sequence WYRGRLGE. For the conjugation of fluorescein, a lysine residue (K) with a primary amine was added at the C-terminus, leading to the sequence WYRGRLGK. In both cases, glycine was added as a linker, for steric reasons. The peptides were synthesized using standard Fmoc solid phase chemistry (SPPS) on Rink Amide AM resin (200-400 mesh, Merck Millipore, Darmstadt, Germany). Each coupling step consisted of a 3 $\times$ 4 min Fmoc deprotection with 20% v/v piperidine in DMF, followed by the coupling using the amino acid (3 eq.), 1-[(1-(Cyano-2-ethoxy-2-oxoethylideneaminoxy) dimethylaminomorpholino)] uronium hexafluorophosphate (COMU, 3 eq.) and *N,N*-Diisopropylethylamine (DIPEA, 6 eq.). The couplings were performed for 2 hours at RT in dry DMF. Between each step, the resin was washed with 3 $\times$ DMF, 3 $\times$ DCM, 3 $\times$ DMF. Following the last deprotection, the peptide was acetylated with 20% v/v acetic anhydride in DMF, 2 $\times$ 15 min.

- WYRGRLGE-dex: the protecting group on the glutamic acid was selectively removed with 1% TFA in DCM, 3 × 10 min. Dexamethasone was conjugated to the peptide on the resin via Steglich esterification. Namely, the resin was incubated with dexamethasone (59 mg, 0.15 mmol, 1 eq.), 4-(Dimethylamino)pyridine (DMAP, 19 mg, 0.15 mmol, 1 eq.) and N,N'-Diisopropylcarbodiimide (DIC, 116 µl, 0.75 mmol, 5 eq.) in DCM/DMF 9:1 at 45°C, overnight. The resin was then washed as usual, followed by 5 × DCM was. The resin was air-dried and the peptide cleaved under reducing conditions (90% TFA, 5% H₂O, 2.5% TIPS, 2.5% EDDT) for 90 min, with shaking at RT. The resin was removed by filtration, and the peptide precipitated in cold diethyl ether. The crude peptide was washed twice with diethyl ether, and let dry.
- WYRGRLGK-FITC: after cleavage from the resin and precipitation in diethyl ether, the peptide was resuspended in DMF, and incubated with fluorescein isothiocyanate (FITC, 20 mg, 0.05 mmol, 0.5 eq.) and triethylamine (20 µl, 0.15 mmol, 1 eq.), overnight at 4°C.

The peptides were resuspended in H₂O/ACN and purified by semi-preparative reversed-phase HPLC (Agilent Prep 100 Å C18, 10 µm, 21.2×250 mm cartridge (PrepHT); 20 ml/min; linear gradient from 20 to 60% solvent B over 20 min (solvent A: H₂O/TFA 999:1; solvent B: ACN/TFA 999:1). Note that the conjugation of dexamethasone considerably extends the retention time. Fractions containing the desired peptide were grouped and lyophilized. The molecular mass of purified peptides was validated by LC-MS (electrospray ionization), using both positive and negative ion modes.

2.6 Fluorescence polarization (collagen binding assay)

Bovine collagen type II (C1188) and collagen type I (6.0 mg/ml, Nutragen 5010, Advanced BioMatrix Inc, San Diego, CA, USA) were used to determine the binding affinity to the WYRGRL peptides. Collagen type II was first diluted in 0.1% acetic acid, pH 3 to prepare the stock solution. The proteins were serially diluted (2-fold) in the assay buffer (100 mM MES, 150 mM NaCl, pH 5.0) containing fixed 750 nM WYRGRLGK-FITC into black 96-well half-area plates (3993, Corning, Wiesbaden, Germany). The final volume in each well was 50 µl. Fluorescence anisotropy was measured on a Synergy 2 microplate reader (BioTek, Winooski VT, USA) using an excitation filter at 485/20 nm and an emission filter at 528/20 nm. The binding constant K_D was determined by plotting the target protein concentration $[P]_T$ versus anisotropy A and fitted using equation (3):

$$A = A_{free} + \left(\frac{A_{bound} - A_{free}}{[F]_T} \right) \times \left(\frac{([P]_T + [F]_T + K_D) - \sqrt{([P]_T + [F]_T + K_D)^2 - 4[P]_T[F]_T}}{2} \right) \quad (3)$$

where A is the experimental fluorescence anisotropy, A_{free} and A_{bound} the anisotropy of the free and fully bound fluorescent peptide, respectively. $[P]_T$ and $[F]_T$ are the total protein (target) and fluorescent peptide (ligand) concentrations, respectively. K_D is the dissociation constant and was extracted from the above formula using non-linear regression with GraphPad Prism 7.

2.7 Chitosan-FITC preparation

Chitosan-FITC was prepared by first dissolving chitosan (C3646) in 1% acetic acid at 1% w/v. This solution was diluted 1:1 in methanol (MeOH). The FITC solution (2 mg/ml in MeOH) was added to the chitosan solution (1:2), and the reaction was allowed to proceed for 3h at RT on orbital shaking. The chitosan-FITC was precipitated by diluting the mixture into 0.2 M NaOH. The precipitate was collected by centrifugation (3,000 g, 5 min) and washed three times with H₂O/MeOH 3:7 until the supernatant was colorless. The precipitate was then resuspended in 0.5 M MES buffer, pH 5.0 at 1% w/v.

2.8 Cartilage diffusion of fluorescein conjugates

Three dye solutions were prepared: 0.25 mg/ml fluorescein sodium salt (Polysciences, Hirschberg an der Bergstrasse, Germany) in PBS, 1.0 mg/ml WYRGLGK-FITC in PBS and 10 mg/ml chitosan-FITC in MES buffer. Ø4 mm punches were cut from bovine articular cartilage and were fixed transversally inside a 1.5ml centrifugation tube. The dye solutions were pipetted onto the punches, sealed and incubated at RT for 24 hours. Following this adsorption (binding) time, the punches were detached from the tubes, cut longitudinally in half, and the cross-section imaged on an epifluorescence microscope (Axio Observer.Z1, Zeiss, Oberkochen, Germany). The half-disks were then transferred to PBS and incubated for an additional 48 hours to wash out weakly-bound molecules. The samples were then cut once more longitudinally (leading to quarters) and imaged on the freshly-cut side. The pictures are shown at a 1X (left) and 10X (right) digital gain to cover a broader range of intensities. Drug retention was calculated with ImageJ v1.51 by measuring the integrated density and normalizing it with the integrated density measured initially.

Chondroitinase digestion: the bovine explants were exposed to 1 U/ml chondroitinase ABC (C2905, Sigma-Aldrich, Buchs, Switzerland) for 90 min at 37°C, with gentle shaking. The buffer was composed of 50 mM Tris, 60 mM sodium acetate, 0.02% BSA, pH 8.0. After digestion, the explants were washed overnight in a large volume of PBS.

2.9 MTS assay

The toxicity of the drug candidates was assessed on human chondrocytes. The chondrocytes were seeded in a 48-well plate at a density of 5,000 cells/cm² in expansion media. After 24 hours, the media was replaced and the cells were incubated with either 1 µM dexamethasone, 10 µM WYRGLGE-dex, 0.1 mg/ml chitosan-dex, 1% DMSO (positive control) or 20% ethanol (negative booking). After three days, the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) substrate was added and kept for three hours. The absorbance was measured at 490 nm using a conventional plate reader.

2.10 In vitro inflammation model

Unless otherwise specified, the media used throughout the study was DMEM (31966, Gibco, Thermo Fisher Scientific, Waltham MA, USA) supplemented with 10 µg mL⁻¹ gentamycin, 82 µg mL⁻¹ L-ascorbic acid 2-phosphate sesquimagnesium and 10% v/v FBS and will be referred to as expansion media. Human chondrocytes were isolated cartilage remnants removed in knee surgery (# KEK-ZH 2013-0097) as previously described.[33] Human chondrocytes from three donors (two women, one man, aged 18 to 38 years old) were encapsulated in alginate hydrogels (Pronova UP LVG, Novamatrix, Sandvika, Norway). The hydrogels were prepared by mixing a cell suspension at 6 × 10⁶ cells/ml at passage 2-3 in expansion media with a pre-warmed alginate solution (2% in 150 mM NaCl) in a 1:1 ratio leading to a final concentration of 3 × 10⁶ cells/ml. The cell suspension was gently homogenized, and 40 µl were quickly pipetted onto the gel caster, which was immersed in sterile, pre-warmed 100 mM CaCl₂ for 30 min. The final gel dimensions were 1.7 ± 0.1 mm in thickness, 5.4 ± 0.2 mm in diameter. Following 24 h culture time in expansion media, the media was changed to low-content FBS (1%) media for 4 hours before the start of inflammation with 1 ng/ml porcine IL-1β. After 18 hours, 0.1 mg/ml chitosan-dex was added or 1 µM dexamethasone as a control. The samples were kept for ten days; at each media change (every three days), IL-1β was supplemented again, but the drugs were not replenished. At the end of the culture, the analysis of inflammatory markers was performed on the supernatant and on the cell lysates. The cell lysates were obtained by lyophilizing the hydrogels and the total protein extracted with RIPA lysis buffer (1% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.7% SDS, 150 mM NaCl, 1mM EDTA, 50 mM NaF, 40 mM Tris-HCl, pH 8, protein inhibitor cocktail (cOmplete™, Roche, Basel, Switzerland), 100 µM Na₃VO₄ (activated) as phosphatase inhibitor). Western blots were performed with an XCell SureLock® Mini-Cell and XCell II™ Blot module (Thermo Fisher, Waltham MA,

USA), according to manufacturer's instructions with anti-COX-2 (polyclonal, 160107, Cayman Chemical, Ann Arbor MI, USA), anti-IL-6 (12153S, Cell Signaling, Danvers MA, USA) antibodies and MMP13 (ab51072, Abcam, Cambridge, United Kingdom).

2.11 THP1 NF- κ B activity assays

THP1 dual cells, were seeded at density 1×10^5 cells/ml in 96-well plates in RPMI 1640 (61870, Gibco, Thermo Fisher Scientific, Waltham MA, USA) supplemented with 10% fetal bovine serum (FBS) and 100 μ g/ml of Normocin and Pen-Strep in an incubator (37°C, 5% CO₂). Following overnight incubation, THP1 dual cells were induced to M0 macrophage differentiation with 15 ng/ml PMA for 24 hours. Upon differentiation, cells were stimulated for 24h with 10 ng/ml LPS (LPS-EK Ultrapure, InvivoGen, Toulouse, France), or 10 ng/ml LPS combined with: 1 μ M dexamethasone, 10 μ M WYRGLGE-dex, 0.1 mg/ml chitosan-dex. Since LPS response is predominantly TLR4-dependent, we used the TLR4 inhibitor CLI095 in order to demonstrate a TLR4-dependent LPS activation. In selected conditions, 1 μ M CLI-095 (InvivoGen, Toulouse, France) was added for 30 min prior to the selected conditions.

THP1 NF- κ B activity was measured using a cell-based reporter gene assay according to the manufacturer's instructions (InvivoGen, Toulouse, France). THP1 dual cells have a stable integrator secreted alkaline phosphatase (SEAP) reporter gene for monitoring nuclear factor (NF)- κ B activation. Upon NF- κ B activation, the SEAP reporter gene are activated, leading to secretion of alkaline phosphatase, and quantifiable by colorimetric assay (QUANTI-Blue, InvivoGen, Toulouse, France) using a spectrophotometer with absorbance at 630 nm.

2.12 Bovine cartilage explant model

Bovine cartilage was obtained from a local butcher, from freshly slaughtered <6 months old calves (n=3). The calf knees were processed within a few hours after animal sacrifice. The cartilage was harvested from the knee condyles, punches ($\varnothing$ 6 $\times$ 2 mm) were cut and washed extensively with PBS supplemented with 50 μ g mL⁻¹ gentamycin and 25 μ g mL⁻¹ amphotericin B. The cartilage plugs were kept at 4°C in PBS containing 0.02% NaN₃ as preservative. The cartilage disks were left in expansion media for one day prior to induction. At the time of induction, media was replaced by FBS-deprived media and explants stimulated with 5 ng/ml porcine IL-1 β . After 18 hours, 1 μ M dexamethasone, 10 μ M WYRGLGE-dex or 0.1 mg/ml chitosan-dex were added to the media, or the punches were left untreated as control. It should be noted that a higher concentration of WYRGLGE-dex conjugate was used to account for its slow release. After 24 hours, the media was changed, IL-1 β supplemented again and the samples were kept in culture for ten days. The media was changed every three days (three times), IL-1 β was added at each media change, but the drugs were never replenished. Finally, the samples were fixed in 4% formaldehyde for 30 min and prepared for histology.

2.13 Biochemical analysis

To complement histology, the supernatant was kept at each media change to quantify glycosaminoglycan loss. 50 μ l of media was mixed with 100 μ l of DMMB solution (50 μ M 1,9-Dimethyl-Methylene Blue zinc chloride double salt (first dissolved in ethanol), 100 mM HCOONa, pH 3.0). The 96-well plate was gently mixed, centrifuged and 100 μ l of the supernatant was transferred to a clean 96-well plate. The absorbance was measured using a microplate reader at 595 nm, and the corresponding GAG amount was calculated. The calibration curve was performed with chondroitin sulfate standards, from 80 μ g/ml to 500 μ g/ml. All the data points are shown in the supporting information.

2.14 Histology

The fixed samples were dehydrated with three steps of increasing ethanol content (20%, 40%, 60%, 45 min/step, in PBS). The samples were then embedded in paraffin using an automated tissue processor

(Milestone Logos J, Sorisole, Italy). Longitudinal cross-sections were obtained by cutting the paraffin-embedded samples with a microtome (HM 325, Microm, Walldorf, Germany) at an 8 μ m thickness. The sections were then deparaffinized with a series of xylene and ethanol steps, with decreasing ethanol content. For GAG staining, samples were first incubated with Weigert's iron hematoxylin working solution for 10 min and washed several times. Then, the sections were incubated in 0.05 % Fast green FCF for 5 min, 1% acetic acid for 10 s and 0.1% Safranin O for 6 min before being dehydrated, mounted and imaged on an automated slide scanner (Pannoramic 250 Flash II, 3Dhistech, Budapest, Hungary). The intensity of Safranin O staining was calculated with ImageJ 1.51 in the following manner. From the colorimetric staining, two channels were extracted corresponding to fast green and safranin O. The area of each sample was determined using the fast green FCF channel (thresholding). The optical density (integrated density) was measured for the Safranin O channel and normalized to each sample's area. For each of the three donors and for each condition, three punches/donor and three sections/punch were used (overall n=27).

2.15 Statistical analysis

Quantitative data are expressed as mean $\pm$ standard deviation. One-way ANOVA using Dunnett multicomparison was performed to compare the intra-cartilage dye retention (Fig. 5) and glycosaminoglycan loss (Fig. 7b). Two-way ANOVA with Dunnett multicomparison was conducted to assess the expression of inflammatory markers (Fig. 6). All statistical analyses were done with GraphPad Prism 7 (La Jolla, CA, USA). Unless otherwise specified, experiments were performed in triplicates and differences were considered significant for $p < 0.05$.

3 Results

3.1 Chemical conjugation for sustained release

The release profile of dexamethasone-loaded materials, chemically cross-linked or physically encapsulated, was studied over one month (**Fig. 2**). Unfortunately, the release from chitosan-dexamethasone was below the detection limit of the system, which can be attributed to the lower substitution degree. To study the release from a polymeric drug carrier, previously published dexamethasone-loaded electrospun poly(ϵ -caprolactone) (PCL) microfibers, which showed a relatively short release half-life (< 2 h), were used as the physical loaded condition (PCL-dex)_{phys}. [33] Dexamethasone (Dex) was chemically cross-linked to PCL and processed in the same way (PCL-dex)_{chem}. In addition, Dex was conjugated to the peptide WYRGLGE, via a hydrolysable carboxylic acid ester linkage. In comparison to physical encapsulation in PCL microfibers, the release half-life was extended 15-fold and 22-fold, for (PCL-dex)_{chem} and WYRGLGE-dex, respectively.

In order to compare enzymatic digestion versus non-enzymatic hydrolysis, the peptide-drug conjugate (WYRGLGE-dex) was incubated with different concentrations of carboxylesterase. Physiologically, concentrations of carboxylesterase are found to reach 0.23 ± 0.11 and 6.80 ± 0.40 U/ml in the whole blood of humans and mice, respectively. [35] Two enzyme concentrations were assessed in this range; 0.1 and 10 U/ml. The lower concentration had no visible effect on the release half-life while the high concentration drastically reduced the release half-life of WYRGLGE-dex.

Fig. 1 (one-column) The release profile of dexamethasone-eluting materials were measured over one month. Drug-loaded poly(ϵ -caprolactone) nanofibers were prepared by physical encapsulation (PCL-dex_{phys} ■) or chemical cross-linking (PCL-dex_{chem} ▲). Dexamethasone was also conjugated to a short peptide, whose hydrolysis rate was measured (WYRGRLGE-dex ◆). Chemical cross-linking allowed the extension of the release half-life of about 20-fold compared to physical encapsulation ($R^2=0.974$ ■, 0.836 ▲, 0.942 ◆, $n=3$). The peptide-drug conjugate was exposed to various concentrations of esterase (◇) to study enzymatic hydrolysis. While the addition of 0.1 U/ml of esterase had no effect on the release half-life ($R^2=0.9132$ ◇, light orange), 10 U/ml of esterase decreased the release half-life of about 3-fold compared to non-enzymatic hydrolysis ($R^2=0.9619$ ◇, dark orange).

3.2 Dexamethasone functionalization and conjugation to chitosan

Unlike PCL, chitosan does not have a carboxylic acid moiety that could be used for conjugation, so primary amines were used instead. Specifically, the primary OH group of Dex was first methacrylated through a nucleophilic acyl substitution. Due to steric reasons, it is largely assumed that the reactivity order among alcohols is primary > secondary > tertiary.[36] Hence, the substitution reaction took place on carbon-21 (*), as depicted in **Fig. 3a**. The successful methacrylation of Dex was confirmed by ^1H NMR, after purification by RP-HPLC (**Fig. 3b**).

Chitosan could be reacted with the methacrylated drug using an iron(III) catalyst.[34] The resulting polymer was analyzed by gel permeation chromatography to confirm successful conjugation (**Fig. 3c**). By simultaneously measuring chitosan signal (214 nm, left axis) and Dex signal (240 nm, right axis, blue), covalently-bound Dex species showed superimposition (t_R 4 min) of the two signals, while unbound Dex elutes later (t_R 16 min). Additionally, there was no noticeable shift in the retention time (t_R) of chitosan, which indicates that the coupling reaction induced no or negligible polymer degradation. It is important to note that although the chromatogram indicates a successful conjugation, the method does not allow the quantification of the substitution degree.

Additionally, the ζ potential of the chitosan-dex conjugates was measured as a function of pH and it was found to reach 18.1 ± 0.7 mV (pH 5.0) and 11.1 ± 0.2 mV (pH 7.4). After freshly filtering the chitosan-dex solutions (0.2 μm), the mean particle size was found to be 12.2 ± 0.9 nm. In comparison, unmodified chitosan was measured at 3.7 ± 0.3 nm. Over time, the polymer chains assembled into bigger particles measuring 470 ± 30 nm (pH 5.0) and large aggregates were formed at pH 7.4. Due to the non-covalent nature of the interactions between the polymer chains and the strong electrostatic force with the cartilage ECM, the prodrugs can diffuse into the full thickness of cartilage. Finally, it should be noted that for uncrosslinked polymers, the measured diameter using this method corresponds to the size of a *spherical* particle having an identical diffusion coefficient.

Fig. 2 (two-column) a) Chitosan-dexamethasone (Chi-dex) was prepared in a two-step procedure. Dexamethasone (Dex) was first methacrylated on its primary alcohol (*), thereby introducing a hydrolysable ester bond. Then, dexamethasone methacrylate (DexMA) was conjugated to the primary amine of chitosan with a Fe(III) catalyst. Dex was then released by hydrolysis, which led to active Dex. b) The methacrylation of Dex was followed by ^1H NMR. Methacrylic protons are highlighted in dark orange while light orange protons correspond to the reference protons. c) Gel permeation chromatography of Chi-dex showed successful conjugation, without polymer degradation. The chitosan signal is plotted on the left Y-axis while the Dex signal is plotted on the right Y-axis (blue). As a negative control, a solution of chitosan and free Dex was used.

3.3 Conjugation of Dex to a collagen type II-binding peptide

The hexapeptide “WYRGRL” was found to have a high nanomolar affinity to collagen type II ($\text{IC}_{50} = 140$ nM) and was used to target polystyrene nanoparticles to cartilage tissue.[31] In the present study, dexamethasone was conjugated to an additional orthogonal glutamic acid residue (E) (**Fig. 4a**). The structure of the conjugated product, WYRGRLGE-dex, was confirmed by mass spectrometry (**Supplementary Fig. 1**). After HPLC purification, the drug loading reaches 100%. With a fluorescent analog, WYRGRLGK-FITC, fluorescence polarization measurements highlighted the affinity and specificity of the peptide towards collagen type II ($K_D = 210 \pm 80$ nM) while it showed no binding to collagen type I ($K_D > 70$ μM) (**Fig. 4b**).

Fig. 3 (one-column) *a*) (Top structure) Structure of the collagen II-binding peptide, before deprotection. (Bottom structure) The glutamic acid residue was selectively deprotected and conjugated to Dex by Steglich esterification, followed by total deprotection, cleavage from the resin and purification. As with chitosan-dex, Dex was released by ester hydrolysis. *b*) Fluorescence polarization confirmed the interaction between the fluorescent analog and collagen type II (orange, $R^2=0.950$, $n=3$) while showing negligible binding to collagen type I (black).

3.4 Dexamethasone infiltration and retention in cartilage explants

After characterization of the drug-carrier conjugates, the infiltration and retention into bovine cartilage explants were assessed. The cartilage punches were exposed, on one side, to the fluorescent analogs (Chitosan-FITC and WYRGLGK-FITC, **Fig. 5**). The microscopy images after binding and washing were quantified, and the amount of fluorophore retained was calculated (**Fig. 5c**). The two drug candidates and free fluorescein control infiltrated cartilage to a similar extent on this timeframe (Supplementary Fig. S3). Small molecules (WYRGLGK-FITC and free fluorescein) infiltrated the cartilage explant in a homogeneous fashion while chitosan-FITC led to a concentration gradient. However, it can be readily seen that only chitosan-FITC led to significant intra-cartilage retention, with over 91% of the initial fluorescein retained (**** $p < 0.0001$). Although considered not statistically significant, the peptide-FITC conjugate retained 8% of the fluorescent dye, which corresponded to a 3-fold increase compared to free fluorescein.

To study the influence of selective enzymatic digestion of the glycosaminoglycans, the cartilage explants were exposed to chondroitinase, before exposure to the fluorescent analogues. Interestingly, this induced a significantly higher fluorophore concentration in the digested regions, after the 24h binding time (**Fig. 5d**). This affected all the drug candidates but was predominant for chitosan-FITC, as shown in **Fig. 5d**. Although there was considerably more binding in the superficial (digested) zone, this did not induce an overall higher retention. This result confirms that (positively charged) chitosan needs negatively charged GAGs to remain bound to the cartilage. The retention of WYRGLGK-FITC remained

unchanged (neither increased or decreased) and confirms that its binding is independent on the glycosaminoglycan network.

Fig. 4 (two-column) *a*) The infiltration and retention of fluorescein-conjugated drug carriers in bovine cartilage explants were quantified. After exposure to the dye solution, the explants of bovine cartilage were transferred to PBS, washed to remove unbound molecules and cut just before imaging. *b*) Cross-section pictures after washing (72h, [†]) of the cartilage disks at 1X and 10X digital gain. The pictures after binding (24h, [†]) are shown in supplementary figure 3. In all images, the diffusion was performed from left (articular surface) to right (subchondral side). Scale bar: 300 μ m. *c*) The amount of dye retained in cartilage was quantified from the micrographs and normalized to the amount of dye initially bound; **** $p < 0.0001$, $n=3$. Chitosan-FITC induced very high drug retention while WYRGLGK-FITC had a lower retention capacity. *d*) Cartilage binding and retention after chondroitinase digestion. Enzymatic digestion of the glycosaminoglycans led to overall more drug infiltration, but not to more retention. This effect was especially important for chitosan-FITC, shown here.

3.5 Toxicity of the drug candidates

In order to assess the toxicity of the drug candidates, we performed a conventional MTS assay on chondrocytes. The chitosan-dex conjugate (Chi-dex) had no cytotoxic effect (Supplementary Fig. S2). However, adding free dexamethasone or the peptide-dex conjugate (WYRGLGE-dex) significantly decreased the cell metabolic activity, to a similar extent (**** $p < 0.0001$). Since there was no significant difference between the latter two, the data suggests that dexamethasone induced this decrease, rather than the peptidic drug carrier.

3.6 Anti-inflammatory effect of chitosan-dexamethasone

To measure the anti-inflammatory potential of chitosan-dex, human chondrocytes were encapsulated into alginate hydrogels. Cells were then stimulated with a pro-inflammatory cytokine, IL-1 β , and treated with a single dose of free Dex, Chi-dex or left untreated. After ten days, COX-2, IL-6 and MMP13 levels were quantified on the cell lysate and culture supernatant (**Fig. 6**). On the time scale investigated, free dexamethasone was able to reduce inflammation of COX-2 but not IL-6, whereas Chi-dex efficiently reduced both markers (* $p < 0.05$, **** $p < 0.0001$). The differences observed between free Dex and Chi-dex were considered statistically significant (** $p < 0.01$, **** $p < 0.0001$) and there was no statistical difference between the non-induced control and the samples treated with Chi-dex. There was already a substantial amount of MMP13 background expression (Neg. control), which was further increased with the addition of IL-1 β . Upon treatment with free dexamethasone and Chi-dex, the level of MMP13 expression was brought back to background expression levels (* $p < 0.05$).

To measure the anti-inflammatory potential of WYRGLGE-dex, human THP1 monocytic cell line was first differentiated into M1 like macrophages. Cells were then stimulated with lipopolysaccharide (LPS),

a classical pro-inflammatory activator, and treated with a single dose of free Dex, WYRGLGE-dex, chitosan-dex or left untreated. In selected conditions, TLR4 inhibitor CLI-095 was added. After 24h, NF- κ B activity was quantified by measuring the content of alkaline phosphatase in the culture supernatant, as the result of the activation of secreted embryonic alkaline phosphatase (SEAP) reporter gene (Fig. 6). After 24h, both free dexamethasone and WYRGLGE-dex were able to significantly reduce the LPS-induced NF- κ B activity (**** $p < 0.0001$). Moreover, WYRGLGE-dex reduction of NF- κ B activity was significantly lower when compared to free dexamethasone alone (* $p < 0.05$), further demonstrating WYRGLGE-dex anti-inflammatory effects. TLR4 inhibition (CLI-095) demonstrated that LPS response was TLR4/NF- κ B dependent and excluded any confounding pathways. To our surprise, Chi-dex reduction of NF- κ B activity was only moderate and failed to reach statistical significant levels (§ $p < 0.07$).

Fig. 5 (two-column) Anti-inflammatory activity. a) Chondrocyte-seeded alginate hydrogels were exposed to a single dose of free dexamethasone (1 μ M) or to the Chi-dex conjugate (0.1 mg/ml), in an inflammatory environment (+ IL1 β). The expression of inflammation markers IL-6, COX-2 and MMP13 was measured in the supernatant as well as in the cell lysate; * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, $n=3$. b) M1-like macrophages were stimulated with LPS and treated with a single dose of free Dex, WYRGLGE-dex, chitosan-dex or left untreated. NF- κ B activity was measured from the culture supernatant as results of NF- κ B activation of secreted embryonic alkaline phosphatase (SEAP) reporter gene; § $p < 0.07$, ** $p < 0.01$, **** $p < 0.0001$.

3.7 Dexamethasone prodrugs prevent GAG loss in a cartilage *ex vivo* model

Finally, the ability to prevent glycosaminoglycan (GAG) depletion was assessed. Bovine cartilage plugs were continuously exposed to an inflammatory stimulus and received a single dose of the drug candidates (a control was left untreated). IL-1 β exposure induced a GAG depletion from the surface, as seen in **Fig. 7a** (arrow). The two drug conjugates were both able to preserve GAG in an inflammatory environment (**Fig. 7b**, left; ** $p < 0.01$, *** $p < 0.001$), while free dexamethasone led to a smaller, statistically non-significant, improvement of GAG depletion. The experiment was performed with three independent donors (**Supplementary Fig. S4a**). The quantification of GAG in the culture supernatant exhibited relatively small differences between IL-1 β -induced and non-induced samples at the early time point (Day 3) (**Supplementary Fig. S4b**, $\Delta=12$ μ g/ml) and increased to reach significant differences at the late time point (Day 10) (**Fig. 7b** right, $\Delta=50$ μ g/ml) (* $p < 0.05$, **** $p < 0.0001$).

Fig. 6 (one-column) a) Bovine cartilage explants were exposed to continuous IL-1 β stress for ten days and treated with one dose of the drug candidates: Dex conjugates (Chi-dex and WYRGRLGE-dex) or free Dex (control). The GAGs were stained with Safranin O, scale bar 200 μ m, n=3. b) (Left) Quantification of the sections in a); ** $p < 0.01$, *** $p < 0.001$, n=9. (Right) The GAGs lost from the tissue were measured in the supernatant. At each media change, the amount of GAG was quantified with a DMMB assay; * $p < 0.05$, **** $p < 0.0001$, n=9.

4 Discussion

The cartilage extracellular matrix consists of an interpenetrating network of negatively-charged glycosaminoglycans (GAGs) and fibrillar collagen type II. In this study, the drug conjugates were designed to target either component. Chitosan, targeting GAGs, or the peptide WYRGRL(GE), targeting collagen type II, were each cross-linked to dexamethasone. It was hypothesized that while electrostatic interactions lead to a high binding efficacy, peptide-protein interactions (i.e., interactions which drive most signaling pathways, composed of van der Waals potential and hydrogen bonding)[37] lead to a highly specific delivery. In fact, intra-tissue retention and therapeutic drug efficacy are intrinsically related. Dexamethasone can only be effective in osteoarthritic conditions when present in the tissue at therapeutic concentrations and for a sufficient time.

Chitosan is a polycationic material that has been widely used in drug delivery and tissue engineering applications. Its biocompatibility is enhanced by a natural degradation mechanism involving lysozyme, an enzyme naturally found in the cartilage extracellular matrix.[38] Chitosan is also thought to stimulate chondrogenesis and synthesis of cartilage ECM to some extent.[38] It is available in GMP clinical grades, can be deacetylated to different degrees and prepared with different molecular weights, making it a tunable candidate for drug delivery in various negatively-charged environments. To date, more than sixty clinical studies are ongoing involving chitosan in a variety of applications, including OA.[39]

The mechanism leading to the binding of cationic molecules to GAGs has several interesting characteristics. Firstly, a sharp electrical potential drop is created at the cartilage-synovial fluid interface, resulting from the high negative fixed-charge density within the tissue.[23] The phenomenon called *Donnan partitioning* causes positively-charged entities to partition within cartilage, while excluding negatively-charged ions, tending to an overall neutral tissue charge.[23] In practice, the number of GAGs is infinitely higher than the number of drug-carrier molecules, leading to a negligible change in overall tissue charge upon drug-carrier infiltration. A solute with a partition coefficient $k = 1$ means that the drug is present at equal concentrations inside cartilage and outside (synovial fluid), while a solute with a partition coefficient $k > 1$ (upward partitioning) implies higher intra-cartilage levels.[23] For example, Avidin has a partitioning coefficient $k = 6$,[29] Neutravidin $k = 0.5$,[29] chitosan

oligomer $2 \leq k \leq 15$ (MW=2.1 kDa, depending on the pH)[40] and chitosan polymer $k > 220$ (MW=57.5 kDa, 70% deacetylation)[40]. A coefficient $k < 1$ (downward partitioning) indicates that, at equilibrium, its concentration within cartilage is smaller than its concentration in the surrounding fluid. Large, sterically-hindered molecules typically have a partition coefficient $k \ll 1$.

Although a single electrostatic interaction is rather weak, the exceptional intra-tissue charge density still induces a strong binding. Assuming that each aggrecan monomer is constituted of 120 chondroitin sulfate monomers[23], each aggrecan molecule can contain more than 6,000 negative charges. The sum of every individual electrostatic interaction not only leads to a strong binding affinity but, since electrostatic interactions are reversible, also leads to a dynamic binding between mobile cationic species and fixed negative GAGs. This dynamic equilibrium prevents the prodrug from being permanently immobilized on the cartilage surface and allows it to reach all the regions of the tissue.[23] Moreover, since the density of negative charges increases with cartilage depth, it creates a stronger attraction to deeper zones of cartilage. Dexamethasone can, therefore, reach the middle and deep zones of cartilage, where the therapeutic drug is most needed.

The binding of positively-charged drug carriers to negatively-charged GAGs is not only based on the net charge, but also on the chemical structure of the carrier. In other words, a high partitioning coefficient does not necessarily guarantee a long retention time.[23] For example, it was found that the small cationic protein Avidin (net charge +6) led to a much long intra-cartilage retention time[29] in comparison with the small cationic peptide Pf-pep (net charge +3), that had a short residency time despite upward partitioning.[41] The dexamethasone-Avidin conjugate also extended the retention time, reduced cytokine-induced catabolism and decreased GAG loss.[42] This interesting result is however mitigated by the detrimental influence of Avidin itself on GAGs, hypothesized by the authors.[30] In comparison, chitosan is a much stronger binder due to its significantly higher net charge and partitioning coefficient. Consequently, the retention time and efficacy of chitosan-dex are expected to be significantly higher, while having high biocompatibility and low cell toxicity.[43]

The other major component of hyaline cartilage, collagen type II, has different levels of organization. At the molecular level, collagen type II assembles into triple helices. The triple helices then form nanofibrils, which, in turn, assemble into thicker collagen fibrils. This highly-organized protein can be targeted, for example, using small peptides. Overall, the number of peptide therapeutics is growing and are proven to be safe and well-tolerated by the body.[44] They are highly selective and can be used unmodified, modified with small molecules to constrain their conformation or as a drug *per se*. [45] Specifically, the peptide sequence WYRGRL was shown to selectively bind to collagen type II.[31] Although peptide-protein interactions are significantly more specific, the low specificity of a chitosan (or Avidin) carrier is not necessarily a negative characteristic, since other joint tissues are also affected by inflammation. There is limited evidence that synovium also contains negatively-charged chondroitin sulfate and could, therefore, be targeted by cationic carriers.[46] However, the synovium is mainly composed of collagen types I, III, V and VI[46], which would prevent drug delivery with WYRGRL. Moreover, in healthy cartilage, glycosaminoglycans form a very dense network that may mask the binding sites on the collagen fibrils, and lead to a lower drug retention. In osteoarthritic cartilage, the loss of GAGs could render the binding sites more accessible to the peptide.

The diffusion of a solute within cartilage and its retention is a multifactorial mechanism depending on several factors. The partition coefficient describes the equilibrium concentration of the solute inside/outside cartilage and largely depends on solute's size and charge, and on the composition of the extracellular matrix.[23] It is believed that the collagen fibril network has a pore size of 60-200 nm [47] and that small carriers with diameters < 10 nm benefit from an enhanced delivery.[23] From this point of view, peptide-drug conjugates are privileged compared to polymer-drug conjugates. The net charge of the drug carrier correlates with its binding affinity. The higher the positive charge, the

stronger the interaction with the negatively charged glycosaminoglycans. From this point of view, chitosan benefits from a very high number of electrostatic interactions. It is important to mention that partitioning and binding are separate processes; a high partition coefficient (high intra-tissue concentration) does not necessarily mean a high retention. From a broader perspective, two driving forces control these mechanisms: kinetics and thermodynamics. A fast kinetics favors partitioning but not necessarily binding, whereas a high enthalpy induces a strong binding without always inducing upwards partitioning.

Alginate, a commonly used hydrogel in the field of cartilage tissue engineering, was chosen to assess the anti-inflammatory potential of Chi-dex. This polysaccharide is composed of negatively charged mannuronate and guluronate monomers, which can bind positively-charged molecules. Two key players of inflammation acting at different levels were quantified: COX-2 and IL-6.[7] Whereas free dexamethasone suppressed COX-2 expression on the timescale investigated, it could not reduce high levels of IL-6, both in cell lysate and culture supernatant. However, when dexamethasone was conjugated to chitosan, it was retained for extended periods of time and led to the downregulation of both inflammatory markers, reaching levels similar to the non-induced control. With the current knowledge of inflammation in osteoarthritis and the unknown exact mechanism of action of glucocorticoids, it is only possible to speculate on the difference between these two markers. COX-2 and IL-6 are both known to be downstream effectors of NF- κ B activation[7], but the effect of Dex on COX-2 seemed to be lasting longer than in IL-6. In previous experiments, it was shown that both cytokines were downregulated after 6 and 24 hours[33], but less is known at later time points (i.e., 240 hours). It is known that dexamethasone acts on glucocorticoid receptors, which affect NF- κ B activation. It can be hypothesized that dexamethasone also has an effect on anti-inflammatory cytokines (IL-4, IL-10, and IL-13) directly affecting COX-2, but not IL-6.[7] This hypothesis is supported by a study in human monocytes where the interactions between anti-inflammatory cytokines and dexamethasone were investigated.[48] It should be noted that the mechanism of action of prodrugs is, by definition, identical to their corresponding drug, after cleavage of the promoiety. Therefore, it is beyond the scope of this work to unravel the complex mechanisms of glucocorticoids.

Osteoarthritis is a disease which affects the whole joint and release of an anti-inflammatory drug from a cartilage 'repository' may benefit several of the affected intra-articular and periarticular tissues. In particular, synovitis or an inflamed synovium is seen in a high number of OA patients and might correlate with faster disease progression.[49] Fat pads, including Hoffa's fat pad in the knee joint, also undergo inflammatory changes during osteoarthritis and are associated with knee pain and sclerotic changes to the subchondral bone are very early changes of the disease.[50] In fact, the inflammatory milieu of the joint caused by circulating pro-inflammatory cytokines could be calmed by off-target effects of dexamethasone released from the cartilage.[51]

Chronic inflammation leads to the progression of osteoarthritis and is accompanied by ECM damage and GAG depletion. Dexamethasone was shown to exhibit chondrogenic properties such as inducing chondrocyte differentiation and proteoglycan biosynthesis[8], in addition to its potent anti-inflammatory effect. Grodzinsky et al. showed that a single dose of dexamethasone limited GAG loss, but it was hypothesized that the low binding and its fast elimination from the joint prevent a long-lasting effect.[3] Bajpayee et al. demonstrated that conjugating dexamethasone to a carrier having a specific affinity for negatively-charged tissues such as Avidin[42] increased its therapeutic efficacy. In the latter study, the authors observed that the Avidin-dex conjugate reduced the extent of GAG depletion induced upon IL-1 stimulation by about 70% over twenty-two days (about 50% after ten days).[42] In the present study, both chitosan-dex and WYRGRLGE-dex conjugates reduced GAG loss by 45% after ten days. Further work is needed to investigate the molecular effects upon binding of dexamethasone to glucocorticoid receptors, and to optimize the dosage and administration frequency

of the drug conjugates. The cellular uptake mechanism has an influence on the efficacy of a prodrug. Most peptides (with exception of cell-penetrating peptides) do not cross the cell membrane efficiently. Therefore, the cleavage between dexamethasone and WYRGRLGE is believed to take place outside the cell, and its cellular uptake to occur via passive diffusion through the cell membrane of the targeted cell, the chondrocytes.[52] The efficacy of dexamethasone can be enhanced through the addition of hydrophobic tails (e.g., clinically-available dexamethasone palmitate). Regarding chitosan, its uptake was shown to be dependent on its molecular weight and some degree of internalization of chitosan-dex can be expected before its intracellular hydrolysis.[53]

In the future, we envision a single intra-articular administration of our drug for treatment of osteoarthritis. Towards this goal, we plan to first perform in vivo experiments in an osteoarthritic rat model. Both the chitosan and collagen-binding prodrugs have advantages. The latter can be made with high levels of purity and precise drug loading, however, its penetration into the tissue is more limited. To increase the retention efficiency, the net charge of the peptide could be increased by the addition of cationic Arg or Lys residues. To target other parts of the joint, the final clinical product could be made of several drug conjugates with different peptide sequences. Chitosan-dex, on the other hand, has a greater intra-tissue retention capability but suffered from a lower drug loading using our method. A higher drug loading could likely be obtained with other conjugation reactions such as EDC/NHS chemistry. Moreover, unmodified chitosan is susceptible to precipitation at pH >5.5. To increase solubility at higher pH, modified forms of chitosan such carboxymethyl chitosan or succinylated chitosan could be considered. Chitosan is also available in a wide variety of molecular weights and deacetylation degrees, which may allow further tuning of the penetration rate into the tissue.

5 Conclusion

Drug-carrier conjugates, or prodrugs, can improve a drug's chemical stability, bioavailability, duration of action and/or solubility.[54] We believe that chemically conjugating dexamethasone to cartilage-targeting carriers can provide enhanced drug infiltration and prolonged retention time within the tissue. In comparison with other drug delivery strategies, the strong asset of this approach is the reversibility of the binding between the drug and its carrier. Although prodrugs require going again through the clinical development phases, drug derivatives do reach the market, as illustrated by dexamethasone palmitate.

In this study, two drug carriers were developed and characterized. It could be shown that while chitosan-dex led to exceptional drug retention within cartilage via electrostatic interactions, WYRGRLGE-dex provided a highly specific but less effective alternative. The latter could be interesting when glycosaminoglycans are depleted from the cartilage (i.e., in end-stage OA) leaving the collagen type II network unshielded. Both drug carriers investigated extended intra-cartilage drug retention in a bovine explant model, downregulated inflammatory markers and reduced GAG loss upon inflammatory stress. Specifically, chitosan is a natural polymer that allows a high degree of tunability (molecular weight and deacetylation degree). Peptides, on the other hand, offer a high degree of substitution, versatility and ready availability of an inexpensive and pure product. Both prodrugs classes show potential for clinical translation and provide a better alternative to the repetitive administration of unmodified glucocorticoids.

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