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Charge based intra-cartilage delivery of single dose dexamethasone using Avidin nano-carriers suppresses cytokine-induced catabolism long term

Ambika G. Bajpayee[1], **Mohiuddin A. Quadir**[2], **Paula T. Hammond**[2], and **Alan J. Grodzinsky**[1,3]

[1]Department of Mechanical Engineering, MIT, 77 Massachusetts Avenue, Cambridge, MA 02139, USA

[2]Department of Chemical Engineering and Koch Institute of Integrative Cancer Research, MIT, Cambridge, MA 02139, USA

[3]Departments of Biological Engineering and Electrical Engineering & Computer Science, MIT, 77 Massachusetts Avenue, Cambridge, MA 02139, USA

Abstract

Objective—Avidin exhibits ideal characteristics for targeted intra-cartilage drug delivery: its small size and optimal positive charge enable rapid penetration through full-thickness cartilage and electrostatic binding interactions that give long half-lives in-vivo. Here we conjugated Avidin with dexamethasone (DEX) and tested the hypothesis that single-dose Avidin-delivered DEX can ameliorate catabolic effects in cytokine-challenged cartilage relevant to post-traumatic OA.

Methods—Avidin was covalently conjugated with DEX using fast (ester) and slow, pH-sensitive release (hydrazone) linkers. DEX release kinetics from these conjugates was characterized using ^{3}H-DEX-Avidin (scintillation counting). Cartilage explants treated with IL-1α were cultured with or without Avidin-DEX conjugates and compared to soluble DEX. Sulfated-glycosaminoglycan (sGAG) loss and biosynthesis rates were measured using DMMB assay and ^{35}S-incorporation, respectively. Chondrocyte viability was measured using fluorescence staining.

Corresponding Author: Alan J. Grodzinsky, MIT Room NE47-377, 500 Technology Square, Cambridge, MA, USA, 617-253-4969 (Phone), 617-258-5239 (FAX), alg@mit.edu.

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Results—Ester linker released DEX from Avidin significantly faster than hydrazone under physiological buffer conditions. Single dose Avidin-DEX suppressed cytokine-induced sGAG loss over 3-weeks, rescued IL-1α-induced cell death, and restored sGAG synthesis levels without causing cytotoxicity. The two Avidin-DEX conjugates in 1:1 combination (fast:slow) had the most prominent bioactivity compared to single dose soluble-DEX, which had a shorter-lived effect and thus needed continuous replenishment throughout the culture period to ameliorate catabolic effects.

Conclusion—Intra-cartilage drug delivery remains inadequate as drugs rapidly clear from the joint, requiring multiple injections or sustained release of high doses in synovial fluid. A single dose of Avidin-conjugated drug enables rapid uptake and sustained delivery inside cartilage at low intratissue doses, and potentially can minimize unwanted drug exposure to other joint tissues.

Keywords

Avidin; electrostatic interactions; dexamethasone; intra-cartilage delivery; cartilage repair; cytokine

INTRODUCTION

Osteoarthritis (OA) affects individual joints making intra-articular (i.a.) therapy a desirable treatment option[1,2], yet simple i.a. injection remains inadequate because drugs are cleared from the joint space rapidly via the lymphatics or vasculature[3,4]. Current research focuses on i.a. injection of drug-encapsulating micro or nanoparticles for slow release of drugs into the synovial fluid[5–7]. However, this approach does not assure penetration of drugs or drug carriers into the dense extracellular matrix (ECM) of cartilage unless a very high drug concentration is used. Such penetration is critical since the majority of chondrocytes and ECM targets reside in the tissue's middle and deep zones. Thus, drug carriers intended for i.a. application should facilitate rapid drug transport into cartilage and remain bound within to achieve sustained intra-tissue therapeutic levels before they are cleared from the joint space.

We previously showed that such challenges of drug delivery into cartilage can be overcome by using cationic nanoparticles less than 10nm in diameter[8]. The high concentration of negatively charged aggrecan inside cartilage offers distinctive advantages for using electrostatic interactions that maximize intra-tissue transport, uptake and binding of cationic drug carrying particles. We used a highly basic protein, Avidin (pI 10.5, net charge +20, 7nm diameter[8]), as an example of a drug carrier, and reported a six-fold upward Donnan partitioning factor at the synovial fluid-cartilage interface (Fig. 1). This resulted in a steep intra-tissue concentration gradient that significantly enhanced Avidin's transport and uptake within various tissues of rabbit[9] and rat knee joints in-vivo[10]. Non-specific electrostatic interactions are weak and reversible and thus allow Avidin to penetrate deep into cartilage. Stronger binding mechanisms can dramatically slow diffusion of drugs or drug carriers and limit their penetration; in contrast, Avidin exhibited weak-reversible binding with intratissue binding sites, allowing diffusion through full thickness of cartilage, yet having a long retention period due to high binding site density of negatively charged proteoglycans.

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Importantly, Avidin demonstrated similar results even after enzymatic removal of 40% GAG in cartilage to mimic early changes of post-traumatic osteoarthritis (PTOA)[8].

Traumatic joint injury, which progresses to PTOA with high incidence rate[11], is characterized by transiently increased synovial fluid concentrations of pro-inflammatory cytokines (e.g., IL-1, IL-6 and TNFα) that are associated with cartilage ECM proteolysis[12–14]. Potential therapeutics, including anti-catabolic glucocorticoids (e.g., dexamethasone (DEX), triamcinolone) and pro-anabolic growth factors (e.g., IGF-1, FGF-18, BMP-7) are known to ameliorate cytokine-induced cartilage degradation in vitro[15–18]. DEX has been shown to suppress cytokine-induced ECM catabolism via GC receptor-dependent pathways[16], and, together with anabolic factors (e.g., IGF-1) can maintain matrix biosynthesis levels[18]. However, in animal[19–21] and human studies[22], ineffective intratissue delivery necessitates high i.a. drug doses which, for glucocorticoids, can cause bone resorption and systemic organ toxicity. Thus, an effective treatment to prevent cartilage degradation associated with PTOA would benefit from delivery of sustained low drug doses *inside* cartilage.

The objectives of this study were to (1) devise a method to conjugate DEX to Avidin and quantify the release of DEX from Avidin-DEX complexes, and (2) test the ability of a single initial dose of Avidin-DEX to inhibit cytokine-induced ECM degradation, rescue cell biosynthesis rates and loss of cell viability in cartilage explants over long durations after penetration/binding of Avidin-DEX into cartilage (depicted schematically in Fig. 1). A combination of fast and slow release chemical linkers for conjugation is used to enable sustained release of DEX inside cartilage. We found that a single dose of Avidin-DEX inhibited cytokine-induced explant catabolism over 3-weeks, while a single dose of soluble DEX had short-lived efficacy and thus required continuous replenishment. Avidin-DEX also inhibited cytokine-induced loss of chondrocyte viability and rescued matrix biosynthesis levels. Avidin has been reported to have no adverse effects on safety in humans[23,24] and no effects on cartilage viability or biosynthesis even at high concentrations in-vitro[10]. Nevertheless, as covalent attachment of poly(ethylene glycol) (PEG) can further shield potential immunogenic responses[25] to highly cationic species, we conjugated PEG to Avidin to further ensure in-vivo compatibility.

MATERIALS AND METHODS

The chemical structures and schematics of the four configurations of Avidin-DEX conjugates synthesized for this study are shown in Fig. 2. Initially, DEX was supramolecularly encapsulated within native (Fig. 2A) and PEGylated (Fig. 2B) Avidin to construct a nanoscale, Avidin-based drug delivery vehicle (abbreviated as **Av+DEX** and **PEG-Av+DEX,** respectively). In addition, to achieve controlled release of DEX from such a nanoparticle delivery construct, we attached DEX to biotinylated PEG through ester (for fast drug release (Fig. 2C)) or hydrazone (for slower and sustained release (Fig. 2D)) linkages, which in turn were supramolecularly coupled to Avidin through the Avidin-biotin interaction. Drug release profiles from these two covalently modified constructs (**PEG-Av-ester-DEX** and **PEG-Av-hydrazone-DEX**) were compared with their non-covalently constructed counterparts (Fig. 2A,B). The biological activity of these constructs was then

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tested using an in-vitro model of cartilage catabolic injury incorporating IL-1α-treated cartilage explants in organ culture.

Supramolecular entrapment of Avidin with ^{3}H-DEX (non-covalent conjugation)

All chemical compounds were purchased from Sigma Aldrich, MO unless specified. Compound **A (Av+DEX**, Fig. 2A) was synthesized by dissolving Avidin in 1xPBS and adding to the solution an amount of unlabeled DEX equivalent to two-times the weight of Avidin used, and an amount of ^{3}H-DEX (specific radioactivity: 100Ci/mmol, concentration: 1mCi/mL, American Radiolabeled Chemicals, MO) equivalent to a final concentration of 5μCi/mL.

For synthesizing compound **B (PEG-Av+DEX,** Fig. 2B), Avidin was PEGylated utilizing its 4 biotin binding sites by mixing 4 molar equivalents of biotinylated PEG-amine (2.3kDa) with 1 molar equivalent of Avidin (Invitrogen, CA) in 1xPBS at room temperature for 2.5h. The fluorescent probe 2,6-ANS (Invitrogen, CA) dye assay was used to confirm stoichiometric binding of biotinylated PEG to Avidin as described [26]. Conjugation of biotinylated PEG to Avidin affects its relative electrophoretic mobility[27]; this was confirmed using SDS-PAGE in 4–12% separating gels (NuPAGE Novex 4–12% Bis-Tris gel System, Life Technologies, CA) under reducing conditions. The gels were then stained for protein (Coomassie brilliant blue). Similar to compound **A**, we added an amount of unlabeled DEX equivalent to two-times the weight of Avidin used, and enough ^{3}H-DEX to yield a final concentration of 5μCi/mL.

The solutions (both PEGylated and native Avidin) were stirred overnight at room temperature, filtered (0.2μm mesh), then ultra-filtered (3.5kDa mesh, spinning at 8,000g for 30min) to remove free DEX from the solution that was not functionalized to Avidin. The final DEX concentration was estimated by measuring radioactivity (microBeta TriLux liquid scintillation counter, Perkin Elmer). Avidin concentration was determined using the bicinchoninic acid (BCA) assay[28]. Drug loading content (DLC) of the final solutions was estimated by:

$$\text{DLC}=\frac{Total\ drug\ encapsulated\ (g)}{Total\ drug\ encapsulated\ (g)+Mass\ of\ Avidin\ (g)}\times 100\%$$

Synthesis of compounds C (PEG-Av-ester-DEX) and D (PEG-Av-hydrazone-DEX)

Compounds **C and D** (Fig. 2) were synthesized by two-step reaction protocols. In the first step, DEX was conjugated to biotinylated PEG through either an *ester* (**3,** Scheme-1, supplementary material) *or a pH sensitive hydrazone linker* (**6,** Scheme-2, supplementary material). In the second step, biotinylated PEG-DEX conjugate was supramolecularly attached to Avidin. (Synthesis steps for both ester and hydrazone linkers are described in Schemes-1 and -2 of supplementary material.)

In-vitro drug release

Release of DEX from Avidin-conjugated DEX compounds was measured using dialysis membranes (Spectra/Por Float-A-Lyzer G2) having molecular weight cutoff 3,500Da. A

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solution of Avidin-conjugated ^{3}H-DEX (5mL) was placed in a dialysis bag immersed in a flask containing 200mL of 1xPBS at pH 7.4 or 4.0 (the latter for hydrazone-linked conjugate; pH adjusted with 1.0N HCl) at 37°C, with continuous magnetic stirring. At different time intervals (starting at t=0), aliquots of solution (50μL) were withdrawn from inside the dialysis bag and the radioactivity measured (liquid scintillation) to estimate the concentration of residual ^{3}H-DEX-Avidin in the dialysis bag. The percent ^{3}H-DEX release was calculated using as follows, where t is the time at which radioactivity is measured and t_o is the initial time:

$$\%\,\text{Drug Released}=\left(1-\frac{radioactivity\,(t)}{radioactivity\,(t_o)}\right)\times 100\%$$

Biological response of cartilage in-vitro

Cartilage disks (3mm diam, 1mm thick) with intact superficial zone were harvested from femoropatellar grooves of 1–2 week old bovine calf knees (Research 87, Hopkinton, MA) as described[8]. Cartilage disks for all treatment groups were matched for depth and location along the joint surface. Disks were equilibrated in serum-free medium (low-glucose DMEM (Cellgro, VA)), 10mM HEPES buffer (Invitrogen, CA), supplemented with 1% ITS (insulin-transferrin-selenium, 10μg/ml, 5.5μg/ml and 5ng/ml, respectively), 0.1mM nonessential amino acids, 0.4mM proline, 20μg/mL ascorbic acid, 100 units/mL penicillin G, 100μg/mL streptomycin, and 0.25μg/mL amphotericin B for two days in a 37°C, 5% CO_2 incubator prior to treatment.

Treatment of cartilage with exogenous cytokine and soluble DEX

To establish the baseline comparison for studying the effectiveness of Avidin-DEX constructs, we first treated groups of cartilage explants with IL-1α (1ng/mL) for 16 days in the presence of the following DEX-treatment conditions: (i) single initial dose of 100nM soluble DEX, and (ii) continuous dose of 100nM DEX. Medium was changed every 2 days and IL-1α was replenished at each medium change. In the 'single DEX dose' condition, cartilage explants were subjected to DEX for only the first 2 days, and subsequent medium changes did not contain DEX, thereby simulating a single i.a. injection of DEX in-vivo. Medium for the 'continuous DEX dose' treatments was, however, replenished with DEX throughout the duration of the 16-day culture.

Treatment with Avidin covalently-conjugated DEX compounds

To determine the concentration of Avidin-conjugated DEX that would effectively suppress IL-1α-induced GAG loss in cartilage with only *one* dose added on day-0 of culture, cartilage explants were treated with or without IL-1α (1ng/mL) and incubated for eight days with one dose of increasing concentrations of PEG-Av-ester-DEX compound (DEX concentrations of 100nM, 4μM and 100μM). Since 4 moles of DEX are covalently conjugated with 1 mole of Avidin using ester linker, these DEX concentrations imply Avidin loading of 25nM, 1μM and 25μM respectively. To test the biological effectiveness of Avidin-conjugated DEX compounds, we treated cartilage explants with or without IL-1α (1ng/mL) for 8, 16 or 22 days in combination with: (i) one-time dose of Avidin-conjugated DEX using ester linker

(PEG-Av-ester-DEX, compound **C**), (ii) one time dose of Avidin-conjugated DEX using hydrazone linker (PEG-Av-hydrazone-DEX, compound **D**), (iii) one time dose of a 1:1 molar ratio of compounds **C** and **D**, (iv) one time dose of soluble DEX. An effective concentration of 100μM DEX was used in all treatments.

Chondrocyte viability in explant culture with Avidin-DEX compounds

Upon termination of culture, 100–200μm thick slices were cut from the center of disks from each treatment condition using established methods[8]. Slices were immediately stained for 2–3 minutes in the dark with Fluorescein Diacetate (FDA; 4mg/ml in PBS) and Propidium Iodide (PI; 40mg/ml in PBS). FDA stained viable cells green and PI stained non-viable cells red[10]. Slices were washed with PBS and imaged (Nikon fluorescence microscope; 4x objective).

Cartilage sGAG loss to medium and sGAG biosynthesis in cartilage

Two days before termination of 8-day cartilage cultures, the medium was supplemented with 5 μCi/mL [^{35}S]-sulfate (PerkinElmer, CT). After 2-day radiolabel period, explants were washed 4 times over 80 minutes with cold PBS to remove free label. Each individual explant was first weighed wet and then digested with proteinase-K (Roche, MN) overnight. Cumulative release of sGAG to the medium and residual sGAG in the digested explants were measured using the dimethyl-methylene blue (DMMB) dye binding assay[29]. Radiolabel in each digested sample and medium standards (^{35}S) were measured (liquid scintillation); radiolabel concentration was calculated from the standards and normalized to explant wet weight.

Statistical Analysis

For all cartilage explant studies, we used the general linear mixed effects model with animal as a random variable, followed by Tukey's Honestly Significant Difference test for comparisons between multiple treatment conditions. There was no effect of animal found and, hence, data across animals were pooled. P-values less than 0.05 were considered statistically significant.

RESULTS

Characterization of Avidin-conjugated DEX compounds

As shown in Fig. 2 the following chemical compounds of Avidin loaded DEX were prepared: (**A**) Av+DEX, (**B**) PEG-Av+DEX, (**C**) PEG-Av-ester-DEX and (**D**) PEG-Av-hydrazone-DEX. Their drug loading content values (DLC, mean±SD) are reported in Table 1. Compounds **C** and **D** have four moles of DEX covalently bound with one mole of Avidin using ester or hydrazone linker; thus, based on molar masses, a theoretical DLC value of 2.3% is expected for fully conjugated Avidin. The molar ratio of biotinylated PEG to Avidin in compounds **B–D** was determined by the fluorescent probe 2,6-ANS dye assay. Addition of biotinylated PEG to the 2,6-ANS dye and Avidin solution displaced the ANS dye, resulting in reduction of fluorescence intensity with increasing molar ratio of biotinylated PEG to Avidin (Fig. 3A). A plateau in fluorescence value was achieved starting at 4:1 molar ratio of biotinylated PEG to Avidin, as expected. Conjugation of Avidin with biotinylated

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PEG also resulted in a shift of molecular weight as observed in SDS-PAGE from the band at ~16kDa (Fig. 3B, monomeric form of Avidin (Av lane) shifted to the broader band at higher MW (PEG-Av lane) due to addition of 2.3kDa PEG chains).

In-vitro DEX release from Avidin

Release profiles of ^{3}H-DEX from non-covalent compounds in PBS at 37°C and pH 7.4 are shown in Fig. 4A. ~70% of loaded DEX was released from compound **A** (Av+DEX) in 3h. PEGylation of Avidin (Compound **B**) did not alter this release rate which was similar to DEX diffusivity in PBS, suggesting absence of any robust binding interaction between DEX and the macromolecular assembly of Avidin or PEGylated-Avidin. In contrast, conjugation of DEX to Avidin via ester linkage dramatically slowed the release of DEX (Fig. 4B, compound **C**), resulting in a half-life of 14.4±1h at pH 7.4 (mean life time=20.8h). This half-life was calculated by fitting a first order exponential decay curve to the measured average concentration inside the dialysis membrane versus time (inset of Fig. 4B).

Conjugation of DEX to Avidin via hydrazone linkage (compound **D**) slowed DEX release even further (Fig. 4C). The hydrazone bond underwent only limited cleavage at pH 7.4, with maximum release at 30% loading. This bond, however, becomes less stable in an acidic environment[30–32]. Consistent with this behavior, DEX release from hydrazone linker at pH 4 showed a mean half-life of 57.4±3.6h. The initial fast release of DEX may be attributed to non-covalently bound DEX on Avidin, implying a longer half-life than 57.4h.

Avidin-DEX compounds did not affect chondrocyte viability in cartilage explants

The cytotoxicity of Avidin-DEX compounds was evaluated by assessing chondrocyte viability in cartilage explants using the live-dead fluorescence assay. Cartilage explants were treated for 48h with compounds **C** and **D**, PEG-Av-ester-DEX and PEG-Av-hydrazone-DEX (final DEX concentration of 100μM). Representative images from 3–4 cartilage disks from all treatment conditions at 48h (Fig. 5B,C) showed minimal cell death and were similar to untreated controls (Fig. 5A). (Some cell death in the superficial zone was typically observed in untreated control explants, depending on the location of harvesting along the joint.)

Effect of single versus continuous dose of soluble DEX on IL-1α treated cartilage

As expected, IL-1α treatment caused increased loss of sGAG-containing aggrecan fragments from cartilage explants over 16 days compared to controls (Fig. 6, $P<0.0001$ all time points). Cumulative sGAG loss was 5–6x higher in IL-1α treated explants compared to untreated controls by day-16. Both single dose (day-0–2) and continuous doses (starting day-0) of 100nM DEX inhibited sGAG loss until day-10 compared to IL-1α alone ($P<0.0001$). However, at day-12, there was a substantial increase in sGAG loss from the single DEX dose explant condition ($P<0.0001$), at the same rate as that from IL-1α treatment alone, while the continuous DEX dose maintained inhibition of sGAG loss at the same rate through day-16.

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Dose dependent bioactivity of Avidin-DEX compounds in cartilage

As shown in Fig. 6, a low but continuous dose of 100nM DEX was sufficient in suppressing IL-1α-induced GAG loss from cartilage explants. To determine the concentration of DEX from one dose of Avidin-delivered DEX that would effectively suppress GAG loss for long times, we tested the dose-dependent response of PEG-Avidin-ester-DEX (compound **C**) on IL-1-treated explants over 8-days (Fig. 7A). We found that one dose of all DEX concentrations (100nm-100μM) in compound **C** could suppress IL-1-induced GAG loss; however, the effect was most prominent using the 100μM DEX-equivalent concentration (Fig. 7A). Hence, a concentration of 100μM DEX delivered through Avidin conjugated DEX structures was chosen for subsequent experiments.

Single dose Avidin-DEX rescued cartilage GAG loss in the presence of IL-1α

Fig. 7B compares cumulative IL-1-induced GAG loss over 22-days in the presence of a single dose of 100μM soluble DEX versus a single dose of 100μM DEX delivered via Avidin (using ester and hydrazone linkers, compounds **C** and **D**). IL-1α treatment increased GAG loss compared to untreated controls at all time points (Fig. 7B, $P<0.0001$). When IL-1α-treated explants were incubated with a single dose of soluble DEX from day 0–2, GAG loss was markedly reduced ($P<0.0001$ compared to IL-1α alone). However, treatment with a single dose of compound **C** (PEG-Av-ester-DEX) suppressed GAG loss even further than soluble DEX alone ($P<0.0001$ compared to soluble DEX, all time points). PEG-Av-hydrazone-DEX inhibited sGAG loss in a manner similar to soluble DEX at earlier time points. While soluble DEX appeared to lose its effect by day 18, PEG-Av-hydrazone-DEX showed more effective inhibition of sGAG loss by days-20 to 22 ($P=0.038$ compared to single dose DEX at day 22). Finally, a 1:1 molar ratio of two linker chemistries with effective DEX concentration of 100μM performed better than the slow-releasing hydrazone linker ($P=0.001$ at day-2, $P<0.0001$, subsequent time points) but similarly to ester linker (Fig. 7B).

Avidin-DEX rescued biosynthesis rates and chondrocyte viability in IL-1α-treated cartilage

Compared to untreated controls, sulfate incorporation was greatly suppressed by IL-1α-treatment in bovine cartilage explants by day 8 of culture (Fig. 8A, $P<0.0001$). Addition of a single dose of 100μM soluble DEX from day 0–2 or continuous dose of soluble 100nM DEX increased ^{35}S-sulfate incorporation compared to IL-1 α alone ($P<0.0001$). One dose of PEG-Av-ester-DEX and PEG-Av-hydrazone-DEX also had similar rescuing effects. In addition, Avidin-conjugated DEX rescued chondrocyte viability in the presence of IL-1α (Figs. 8Bi–vi). A dramatic increase in cell death was observed after treatment with IL-1 alone by day-8 (Fig. 8Bii). In contrast, addition of a single dose of 100μM DEX on day 0–2 (Fig. 8Biii) or a continuous dose of 100nM DEX (Fig. 8Biv) successfully prevented cell death induced by IL-1α. Treatment with a single dose of PEG-Av-ester-DEX or PEG-Av-hydrazone-DEX (Figs. 8Bv–vi) added on day 0–2, also successfully rescued chondrocyte death similar to that observed using the soluble DEX conditions.

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DISCUSSION

This work demonstrates a drug delivery approach to long term suppression of cytokine-induced loss of GAG and chondrocyte viability inside cartilage with just a single-dose treatment exemplified using Avidin-conjugated DEX. Avidin, due to its ideal size and positive charge, rapidly penetrates through full thickness cartilage due to electrostatic interactions and reversible binding within tissue, resulting in long half-lives in-vivo[9,10]. We used a combination of fast (ester) and slow release-pH cleavable (hydrazone) linkers to conjugate DEX with Avidin and compared its biological efficacy with a single dose of soluble DEX in an IL-1α-challenged cartilage explant model. IL-1α is one of the family of inflammatory cytokines that is thought to play a critical role in the initial events associated with progression to PTOA[14] and causes GAG loss by up-regulating ADAMTS-4,5 in bovine[33] as well as human[14] cartilage. We used DEX as an example drug as it has been previously shown to block cytokine-induced cartilage degradation[16,18].

Our data show that a single dose of DEX alone could not provide long term suppression of GAG loss compared to continuous DEX treatment during culture. Removal of 100nM DEX after day 0–2 provided inhibition of GAG loss only for the first 8 days (Fig. 6), after which increased aggrecan catabolism continued, such that the cumulative GAG loss by day 16 was 1.8x higher than the continuous DEX dose condition. However only a single dose of either PEG-Av-ester-DEX or compound **C**+**D** (1:1 molar ratio of ester and hydrazone) added at day 0–2 was needed to significantly reduce IL-1-induced GAG loss compared to the soluble DEX condition over 3-weeks (Fig. 7B). Compound **D** had a similar effect as 1-dose soluble DEX at earlier time points, but by day-22, a significantly greater suppression of GAG loss was observed. In separate experiments, there was negligible uptake of Avidin by the chondrocytes (Fig S1 Supplementary Material). Thus, we conjugated DEX to Avidin using hydrolysable linkers like ester and hydrazone to facilitate release of DEX, making it available for uptake by chondrocytes.

The ester (compound **C)** is a fast-releasing linker giving a mean half-life of 14.4h at physiological conditions (Fig 4B), suggesting that 70% of the loaded DEX released before the first medium change (48h after start of culture), thereby providing an initial appropriate dose of DEX to chondrocytes in cartilage that is critical for inhibiting IL-1α-induced catabolic effects. Hydrazone is a slow releasing linker with a mean half-life of 57.4h at low pH4 (Fig 4C). As it cleaves in acidic environments[34], hydrazone should release DEX closer to negatively charged GAG chains in the chondrocyte pericellular matrix (where aggrecan concentration is highest[35]). Additionally, a decrease in pH from 7.4 to 5.5 was reported at cartilage surfaces of OA patients[4]. At the micro-scale aggrecan density increases with depth into cartilage from the superficial zone[36]. Thus, as Avidin carries the drug into middle-deep zones of cartilage where most chondrocytes reside, the rate of drug release from hydrazone linker should increase with depth into cartilage. A combination of ester and hydrazone is expected to yield greater rescuing effect at longer times in-vivo, and this approach is currently being tested in a rabbit anterior cruciate ligament transection (ACLT) model of PTOA.

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Treatment with Avidin-DEX compounds did not cause cytotoxic effects as there was no effect on chondrocyte viability (Fig. 5). Both compounds rescued cytokine-induced decrease in biosynthesis and chondrocyte viability, similar to soluble DEX conditions (Figs. 8A–B). Previously, we showed that Avidin alone, up to 100μM concentration, did not affect chondrocyte biosynthesis of proteins and proteoglycans in similar-aged bovine cartilage explants[10]. Here, we used one dose of <25μM Avidin for delivering 100μM DEX to cartilage explants in low glucose medium containing 1% ITS. Therapeutic use of Avidin can elicit the production of anti-Avidin antibodies in-vivo[23,37,38] but this has been shown to have no effect on its safety or efficacy in humans[23,39]

A limitation of this study is that experiments were performed using culture medium without synovial fluid (SF). Hyaluronic acid and other negatively charged moieties in SF could in principle lead to binding of Avidin, preventing penetration of Avidin and conjugated drugs into cartilage. However, as we reported previously, i.a. injection of Avidin into rabbit[9] and rat[10] knee joints *in vivo* resulted in Avidin penetration from knee SF into the superficial zone and through the full thickness of joint cartilages, as documented by confocal images[10] and by direct measurement of the half-lives of Avidin inside these knee cartilages[9,10]. Additionally, in separate tests, we observed that the release of DEX from PEG-Av-ester-DEX and PEG-Av-hydrazone-DEX after 100 h in bovine SF *in vitro* is similar to that found in the PBS conditions of the present manuscript. We therefore suggest that Avidin-DEX compounds can indeed be carried into cartilage *in vivo*, and are currently testing this approach in a rabbit ACLT model.

In summary, Avidin as a nano-carrier can rapidly transport candidate DMOADs into the middle and deep zones of cartilage and bind reversibly inside the tissue, thereby creating a drug reservoir and providing sustained delivery to nearby cell and ECM targets. It is now accepted that multiple DMOADs may be useful for disease modification[15]. One example of such a drug combination would be a pro-anabolic growth factor (e.g., IGF-1[17,40]) known to stimulate cartilage repair, and an anti-catabolic glucocorticoid (e.g., DEX[18]). Growth factors such as IGF-1 can be similarly conjugated with Avidin by using a combination of slow and fast drug releasing chemical linkers to enable targeted and sustained delivery inside cartilage along with glucocorticoids.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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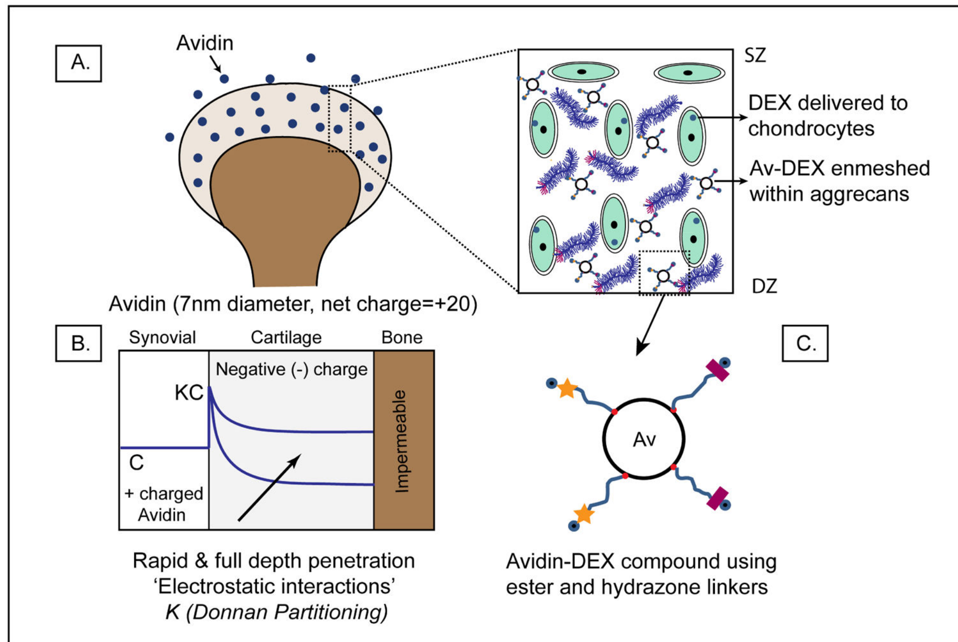

Fig. 1.

Schematic of the concept underlying the drug delivery scheme developed. **(A)** Avidin nanoparticles due to their ideal size and high positive charge can penetrate through full thickness of cartilage owing to weak and reversible electrostatic binding with the negatively charged aggrecan within cartilage. **(B)** A high upward Donnan partitioning factor enables rapid transport into and within the cartilage[8]. **(C)** A single dose of Avidin-conjugated dexamethasone (DEX) using fast (ester) and slow release linkers (hydrazone) enabled delivery of DEX to chondrocytes which suppressed cytokine-induced loss of sGAG and chondrocyte viability. SZ=superficial zone of cartilage, DZ=deep zone

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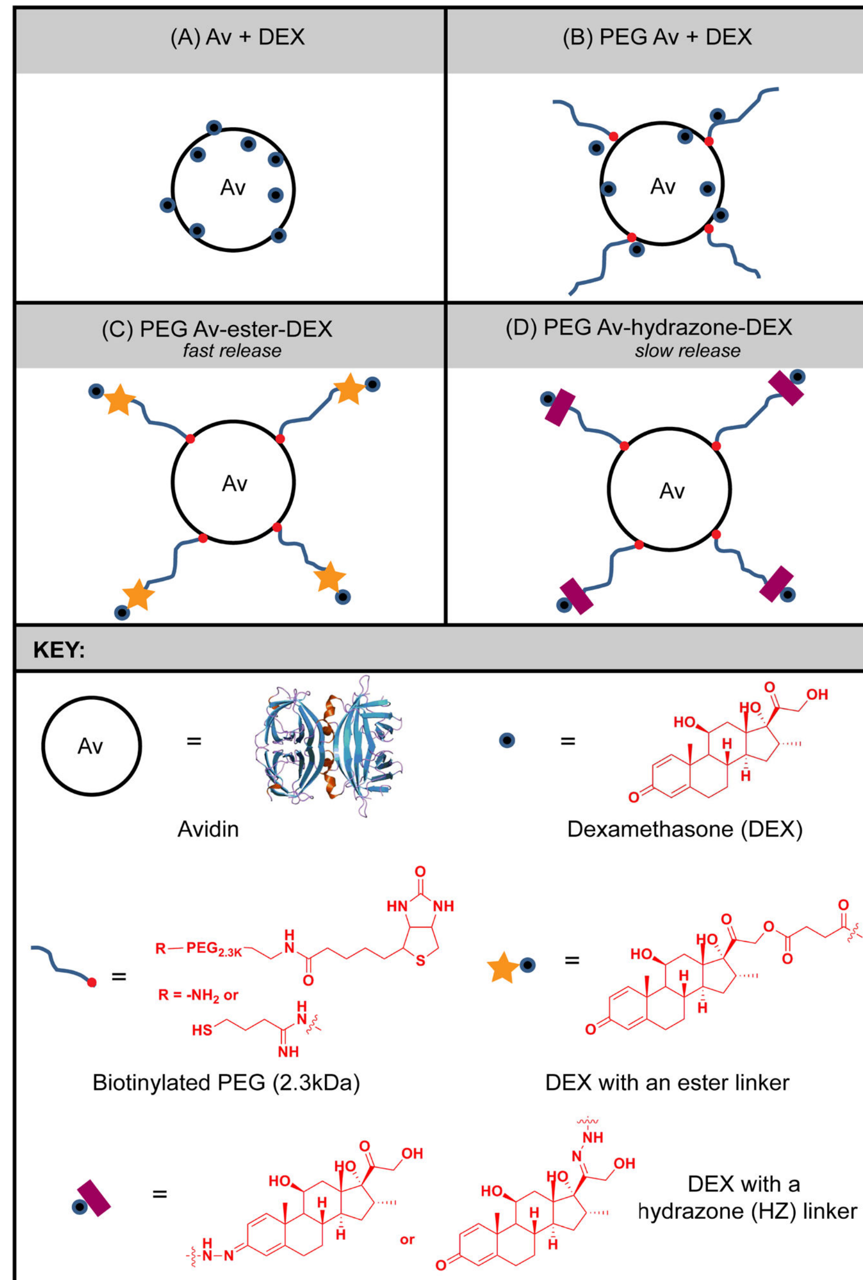

Fig. 2.

Schematic representation and chemical structures of the four Avidin (Av) conjugated dexamethasone (DEX) compounds formulated. **(A)** Av+DEX: Avidin supramolecularly (non-covalently) loaded with DEX. **(B)** PEG-Av+DEX: PEGylated Avidin supramolecularly (non-covalently) loaded with DEX. **(C)** Fast release PEG-Av-ester-DEX: PEGylated Avidin covalently conjugated with DEX using ester linkers. **(D)** Slow release PEG-Av-hydrazone-DEX: PEGylated Avidin covalently conjugated with DEX using pH sensitive hydrazone (HZ) linkers.

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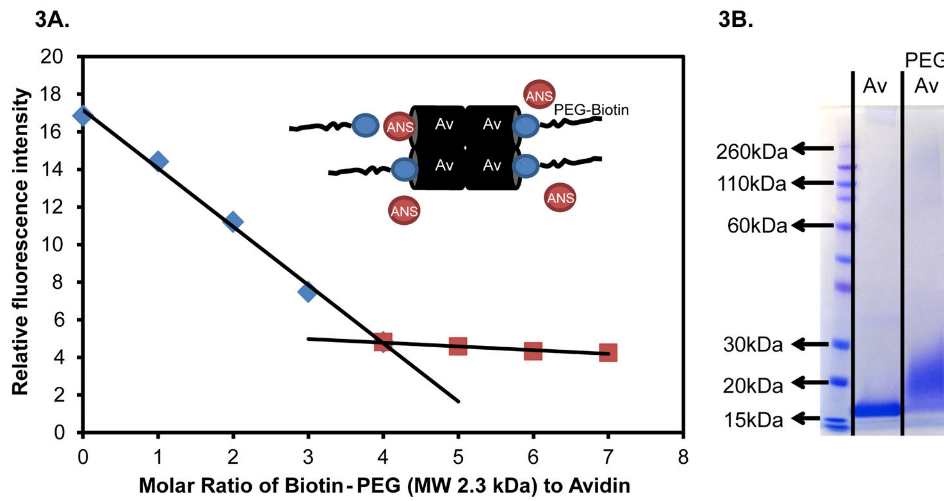

Fig. 3.

(A) Titration curve of 2,6 ANS-fluorometric assay for stoichiometry of biotin-PEG (2300 Da) with Avidin in PBS buffer at pH 7.4. **(B)** SDS PAGE (4–12%) of Avidin (Av) and PEGylated Avidin (PEG Av) under reducing conditions stained with Coomassie Blue

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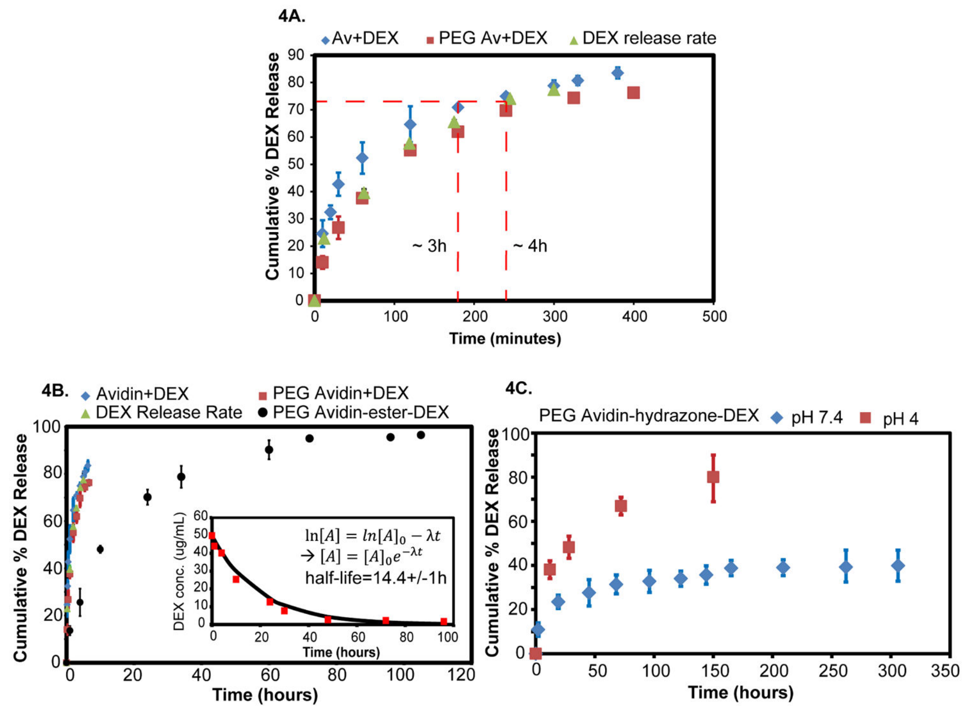

Fig. 4.

(A) In vitro ^{3}H-DEX release profiles for non-covalently DEX loaded Avidin compounds compared with diffusivity of ^{3}H-DEX through the dialysis membrane in PBS (pH 7.4) at 37°C. **(B)** In vitro ^{3}H-DEX release profile for PEG-Av-ester-DEX in PBS (pH 7.4) at 37°C. Ester, a fast release linker, resulted in a mean half-life of 14.4h. $A(t) = A_0\exp(-\lambda t)$, where $A(t)$ is the DEX concentration at time t, A_0 is the initial DEX concentration inside the dialysis membrane at t=0 and $1/\lambda$ is the characteristic exponential decay time. The half-life ($t_{1/2}$) is calculated as $t_{1/2}=\frac{\ln(2)}{\lambda}$. **(C)** ^{3}H-DEX release profiles for PEG-Av-hydrazone-DEX in PBS at 37°C at pH 7.4 (diamonds) and at pH 4 (squares). Hydrazone is an acid cleavable linker, and hence resulted in a slow release of DEX in acidic environment (pH 4) with a mean half-life of 57.4h. Data is from 4–5 DEX release experiments and are presented as Mean ± 95% confidence interval.

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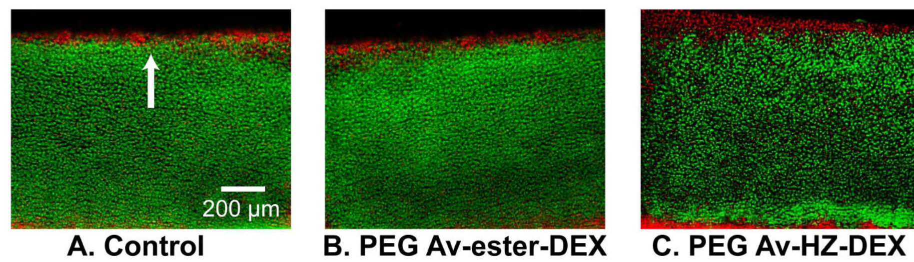

Fig. 5.

Images of fluorescently stained bovine cartilage explants to check for chondrocyte viability after 48h incubation with **(A)** basal media, untreated control (B) PEG Av-ester-DEX and (C) PEG Av-hydrazone-DEX. Effective DEX concentration of 100µM was used. Green indicates viable cells and red indicates non-viable cells.

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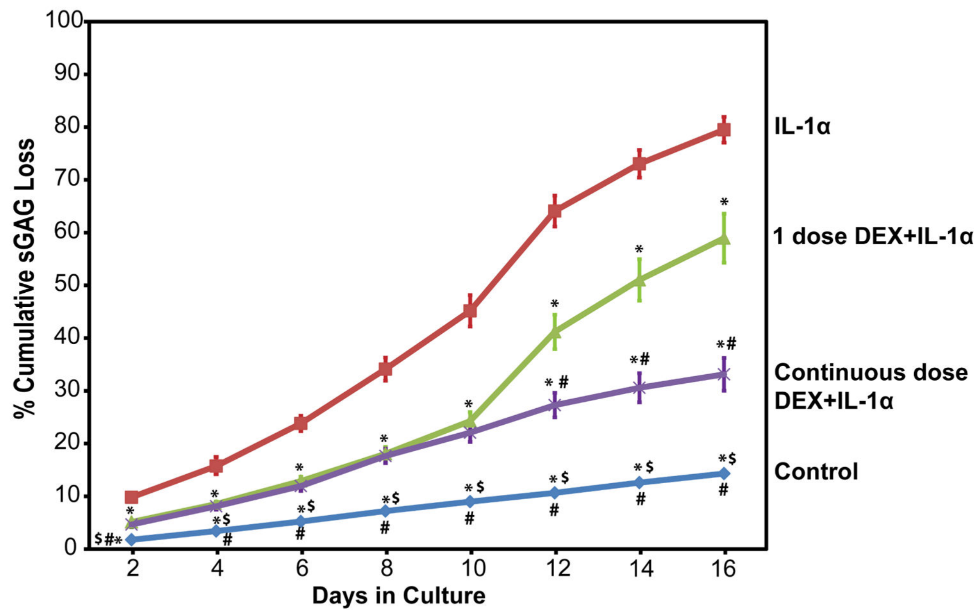

Fig. 6.

Effect of one dose vs. continuous dose of 100nM DEX on IL-1α simulated GAG loss in bovine cartilage explants. Cartilage tissues were cultured with or without IL-1α (1ng/mL) and with one time dose of 100nM DEX or continuous dose of 100nM DEX for 16 days. Data are presented as Mean ± 95% confidence intervals, N=3 animals, n=6 explants for each treatment condition from each animal (18 explants per condition). All treatment conditions are significantly different from the untreated control. Error bars for the untreated control data points are small and thus not visible. * vs IL-1α alone, # vs one dose DEX, $ vs continuous dose DEX, P<0.0001.

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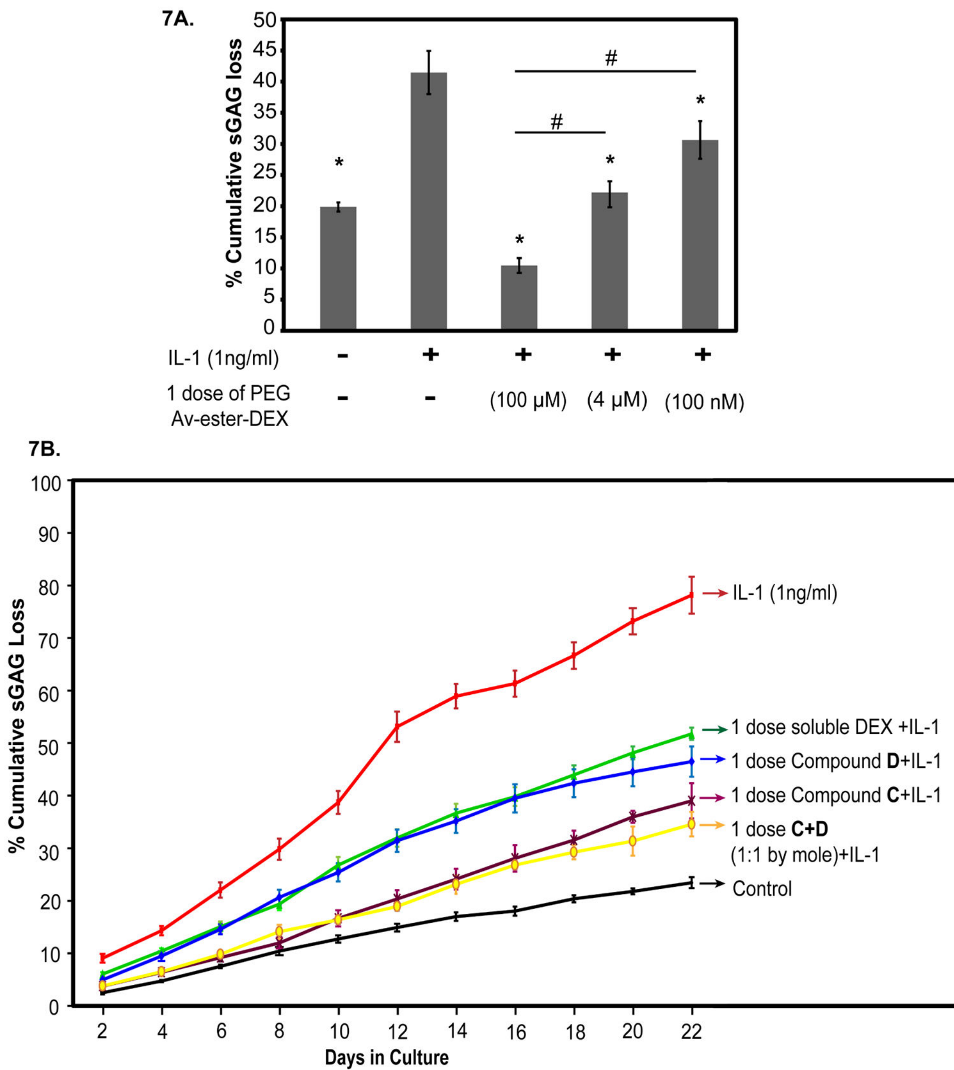

Fig. 7.

(**A**) Effect of one dose of compound **C** (PEG-Av-ester DEX) on cumulative sGAG loss in IL-1α (1ng/ml) treated bovine cartilage explants at day 8 of culture period. Effective DEX concentration in these compounds is shown in brackets (100nM to 100μM). Data are presented as Mean ± 95% confidence interval, N=2 animals, n=6–8 explants each treatment condition. * vs IL-1α alone, # vs 100 μM DEX, P<0.0001

(**B**) Effect of one dose of effective DEX concentration of 100μM in cumulative sGAG loss in IL-1α (1ng/ml) treated bovine cartilage explants over a period of 22 days. One dose of DEX was provided in form of soluble DEX (green), compound **C** (PEG-Av-ester DEX, purple), compound **D** (PEG-Av-hydrazone DEX, blue) or 1:1 molar ratio of compounds C and D (yellow). Black line shows cumulative sGAG loss in untreated control cartilage and the red line shows the sGAG loss in IL-1α alone treated cartilage over a period of 22 days. Values are Mean ± 95% confidence interval, N=4 animals, n=6–12 explants each condition from each animal. All conditions are significantly different from IL-1 alone (red) at all time points (P<0.0001). Soluble DEX (green) and compound D (blue) are significantly different

from control (black) starting at day 4 (P<0.0001). Compound C (purple) and the combination C+D (yellow) are significantly different from control (black) starting at day 10 (P<0.0001) and day 8 (P=0.001 at day 8 and P<0.0001 starting day 10) respectively.

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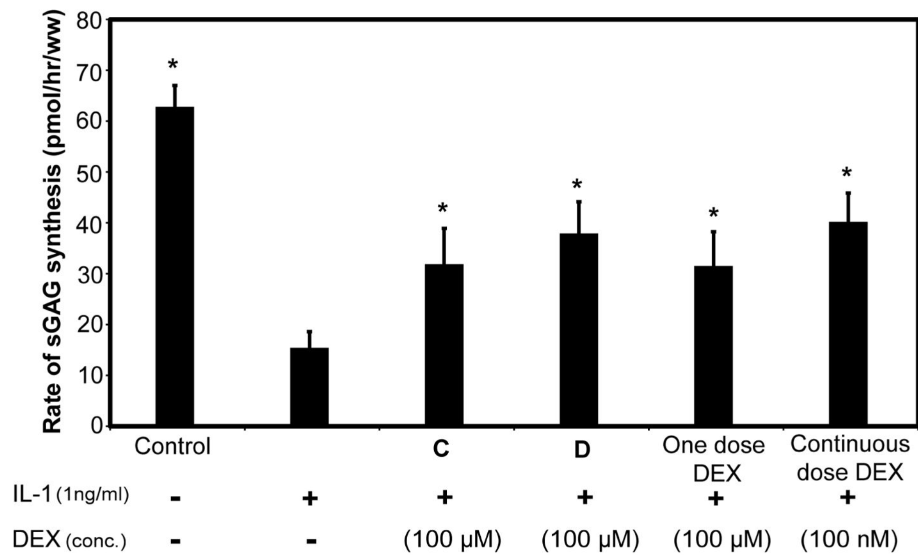

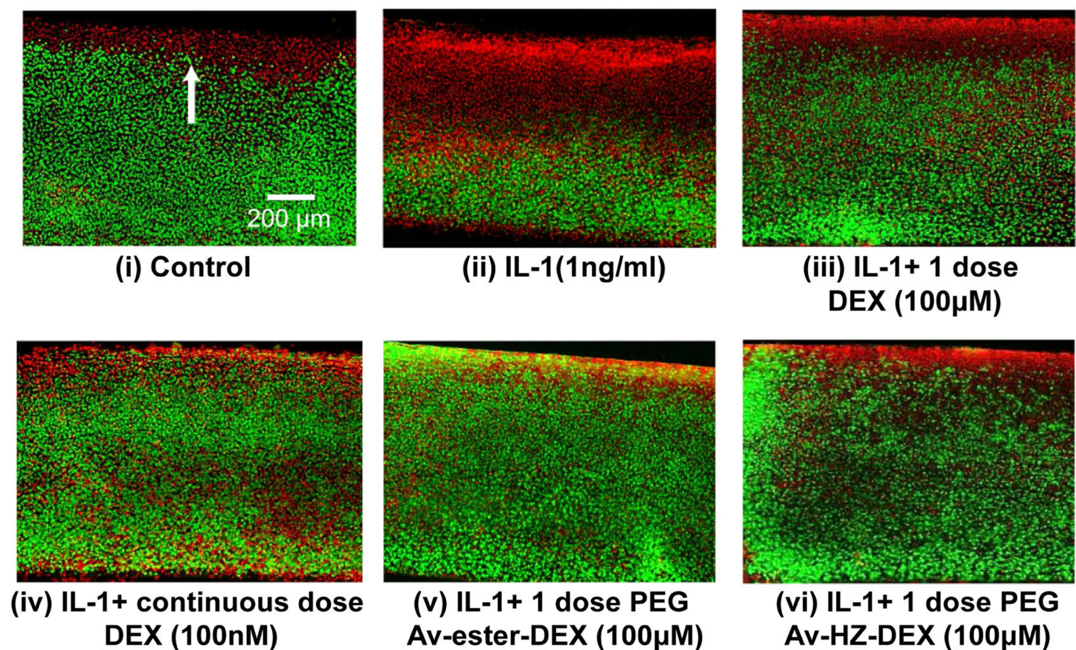

Fig. 8.

(A) Rate of sGAG synthesis during the last 48h of the 8 days culture time period normalized by the wet weight of cartilage explants. Data are presented as Mean ± 95% confidence interval. N= 4 animals, n=6 explants each condition from each animal. * vs IL-1α alone, P<0.0001. One dose of compounds C, D and DEX (effective DEX concentration of 100 μM) are compared to continuous dose of 100nm DEX.

(B) Images of fluorescently stained bovine cartilage explants (4x objective) cultured for 8 days to check for chondrocyte viability in **(i)** basal medium, untreated control **(ii)** treated

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with 1ng/ml of IL-1α alone. The following conditions were treated with IL-1α (1ng/ml) along with **(iii)** 1 dose of 100μM soluble DEX **(iv)** continuous dose of 100nM soluble DEX during 8 days of culture **(v)** 1 dose of PEG-Av-ester-DEX and **(vi)** 1 dose of PEG-Av-hydrazone (HZ)-DEX (final DEX concentration of 100μM in (v) and (vi)). Green indicates viable cells and red indicates non-viable cells. The top edge of each image (arrow) shows the superficial zone and the bottom represents the transected middle/deep zone. Scale bar =200μm

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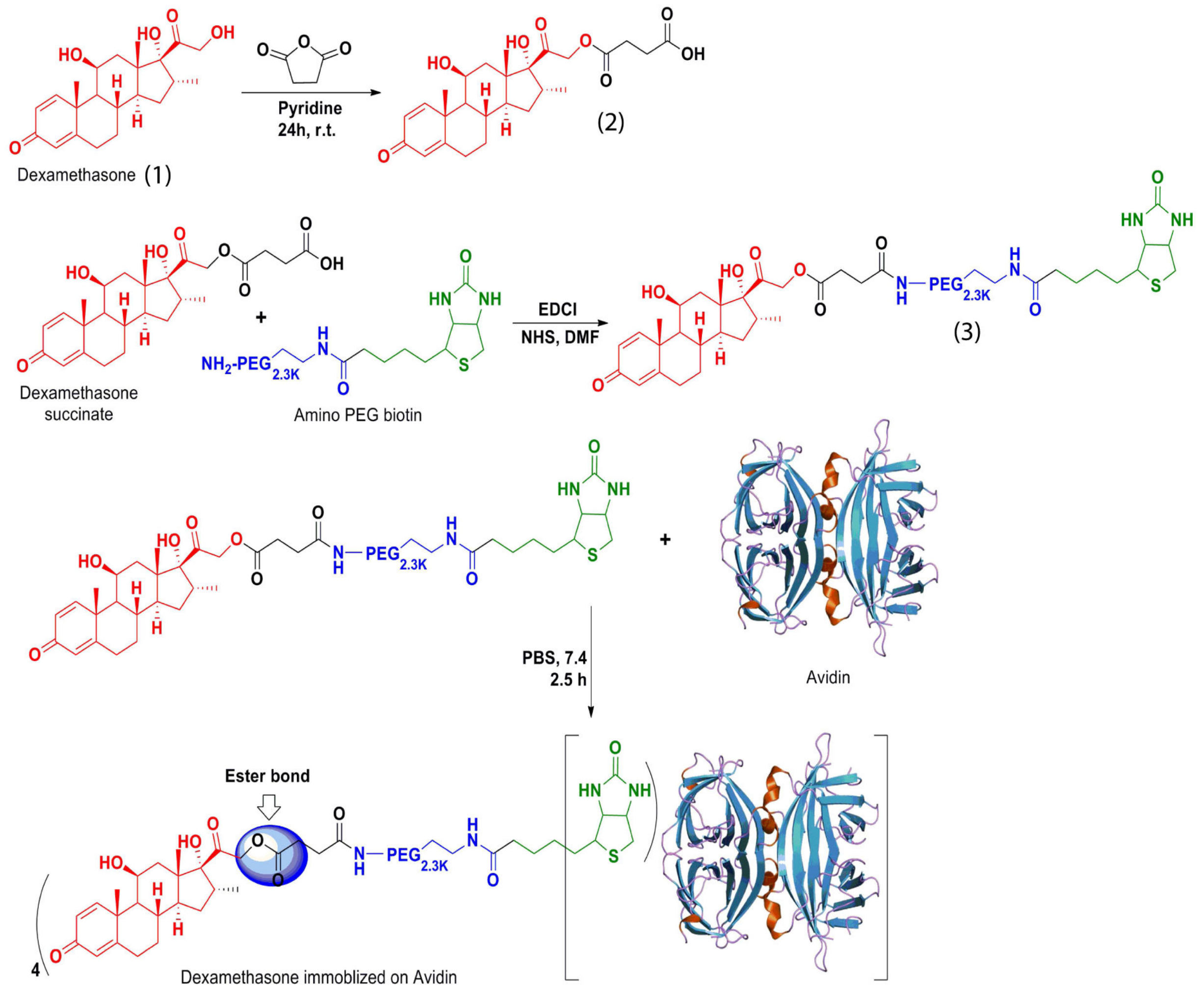

Scheme 1.

Steps of synthesis for covalent conjugation of Avidin with PEGylated dexamethasone using ester linker

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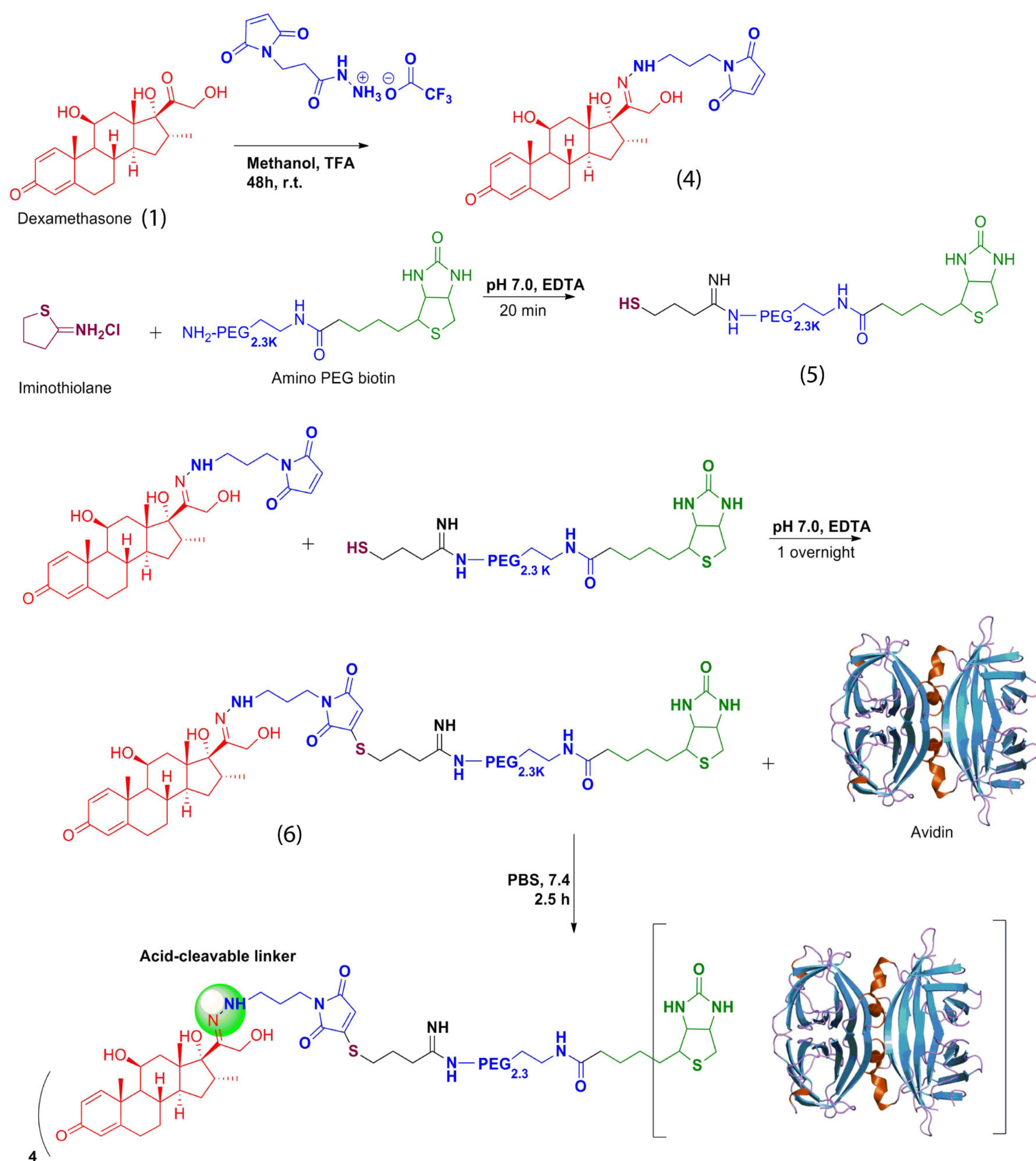

Scheme 2.

Steps of synthesis for covalent conjugation of Avidin with PEGylated dexamethasone using hydrazone linker

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Table 1

Drug loading content (Mean+/−SD) for Avidin conjugated DEX compounds

Compound	Compound type	DLC (wt%)
A	Av + DEX	33.2 ± 4.8
B	PEG-Av + DEX	32.8 ± 3.6
C	PEG-Av-ester-DEX	2.2 ± 0.3
D	PEG-Av-hydrazone-DEX	1.9 ± 0.1