SYNTHESIS OF CARTILAGE TARGETING MULTI-ARM AVIDIN AS A CATIONIC DRUG CARRIER

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ABSTRACT

Osteoarthritis (OA) is a debilitating disease of joints that affects soft tissues like cartilage. Intra-articular (IA) delivery of drugs to cartilage remains a challenge due to their rapid clearance from the joint and hindered transport into the cartilage due to its complex and dense architecture containing a high density of negatively charged aggrecans and a meshwork of Collagen Type II. Avidin, due to its optimal size and net positive charge (diam ~7nm, z=+14 mV), was shown to rapidly penetrate through the full thickness of cartilage due to electrostatic interactions, bind with the negatively charged aggrecans and provide a sustained release of drugs to the chondrocytes thereby converting cartilage from a barrier to drug entry into a drug depot. Additionally, Avidin was found to remain bound within the rabbit cartilage even after 3 weeks following its IA administration in a rabbit ACL transection (ACLT) model of post-traumatic OA (PTOA). Avidin was conjugated with four 2.3kDa PEG-Biotin using Avidin/Biotin reaction and a small molecule anti-inflammatory drug, dexamethasone (Dex). This design had 4 moles of Dex covalently linked per mole of Avidin. A single IA dose of Avidin-Dex effectively rescued injury induced catabolism in a rabbit ACLT model significantly better than free equivalent Dex dose (0.5 mg) alone further reinforcing that a cartilage depot delivery of drugs is an effective way of OA treatment. This Avidin-Dex nano-construct design, however, required a high dose of Avidin (20 mg) to deliver 0.5 mg of Dex that was associated with loss of some glycosaminoglycans (GAGs) from cartilage owing to drop in osmotic swelling pressures. To use Avidin as a cartilage targeting nanocarrier, it is important to redesign the nano-construct to increase its drug loading content. Here, we design a novel multi-arm Avidin nano-construct that can provide 7x higher amount of Dex loading while keeping the net size and charge of the construct similar to that of native Avidin’s and not significantly affecting its intra-cartilage transport properties like rate of penetrability, uptake, and retention.
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-Tengfei He
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1. Introduction

Osteoarthritis (OA) is a chronic inflammatory joint disease affecting knees, hips, fingers, and low spinal regions. More importantly, it is one of the most disabling diseases in developed countries with an estimated social cost of between 1 % and 2.5 % of gross domestic product (GDP) [1]. OA not only lowers quality of life, but also is a huge economic and social burden to the society. Various kinds of risk factors can contribute to OA onset and progression including age, obesity, joint trauma, gender, genetics, metabolism and diseases like diabetes [2, 3]. Despite its wide prevalence, OA remains without a cure.

Acute mechanical injuries like ACL and meniscus tears can induce post traumatic osteoarthritis (PTOA) that largely affects young active people. 50-80% of patients with a traumatic joint injury progress to OA like symptoms within 8-10 years [4]. A mechanical injury initiates catabolic biological pathways that disturb the joint homeostasis resulting in degradation of cartilage and other joint tissues [5]. Inflammatory cytokines like IL-1, IL-6 and TNFα are elevated inside the joint right after an injury that induce production of aggrecanases and metalloproteases, which degrade cartilage matrix components like collagen type II and aggrecans [6, 7]. The effect of this catabolic response can be prevented by intervening early on (ideally right after an injury) [8] by using drugs that can inhibit the associated inflammatory responses, prevent cell death and cartilage matrix degradation as well as promote synthesis of new matrix components [5]. Dexamethasone (Dex), a potent synthetic glucocorticoid (GC), has been shown to switch off the
inflammatory genes in the cells through GC pathways which is able to triggered by pro-inflammatory cytokines as mentioned before [9]. Lots of works reported the application of Dex for suppressing the inflammatory cytokines. In an in-vitro experiment, bovine and human cartilage explants have been treated with TNF-α and IL-6 together to induce the inflammatory reaction in the cartilage. Then continuous dose of 10 nM Dex was administered following cytokine treatment resulting in significant suppression in gag loss from bovine cartilage. [6]. Then in an in-vivo experiment, a high dosage of Dex was injected into a PTOA rabbit model every three days for 3 weeks. Results showed a significant suppression in joint inflammation[8]. In addition, sustained doses Dex and IGF-1 (a pro-anabolic growth factor) have been used in combination and shown to suppress cytokine induced catabolic activity as well as enable matrix biosynthesis [7]. In a recent clinical trial, ACL tear patients received an intra-articular (IA) injection of 40 mg triamcinolone acetonide (TCA), a GC similar to Dex, within 4 days and 10 days. Data showed that early intervention with GCs and repeated dose can suppress the expression of carboxyl-terminal telopeptides of type II collagen released in the serum more efficiently than the ACL patients without any injection [10]. Although repeated doses of GC were required for the effective treatment of PTOA, high GC doses are associated with severe adverse side-effects, like bone desorption and systemic organ toxicity [11, 12]. Recently, some unsuccessful clinical trials showed that inhibitors of IL-1 failed to suppress the inflammation caused by OA, due to their inability to penetrate into the cartilage, and reach the chondrocytes [13, 14]. Therefore, a drug delivery system that can provide sustained low drug dose over
several weeks inside cartilage is critical for preventing the cartilage inflammation related to PTOA.

Cartilage due to its dense extracellular matrix (ECM) containing aggregans with a high density of negatively charged glycosaminoglycan (GAG) chains and type II collagen hinders penetration of drugs and most macromolecules into its middle and deep zones where a majority of chondrocytes reside [15]. The high density of negatively charged groups, however, provides a unique opportunity to use electrostatic interactions for enhancing transport rate, uptake and retention of positively charged drugs/drug carriers [16, 17]. Previous work by Bajpayee et al. showed that a highly positively charged glycoprotein, Avidin (pl 10.5, 7 nm diameter), rapidly penetrated through the full-thickness of cartilage resulting in 400x higher intra-cartilage uptake than its electrically neutral counterpart, NeutrAvidin [15]. Avidin retained inside the cartilage for several weeks while NeutrAvidin desorbed out rapidly within 24 h. Avidin was then covalently linked with 4 moles of Dex (Av-Dex) using hydrolysable ester linkers that was shown to suppress IL-1α induced catabolism significantly greater than the Dex alone in an in-vitro cartilage explant culture model of PTOA [18]. A single low dose IA injection of Av-Dex (0.5 mg Dex) suppressed injury induced joint inflammation, synovitis, incidence of osteophyte formation and restored trabecular properties at 3 weeks significantly greater than free Dex alone in a rabbit anterior cruciate ligament transection (ALCT) model of PTOA [19]. This Av-Dex nano-construct design, however, required a high dose of 20 mg Avidin (300 μM) to deliver 0.5 mg of Dex that resulted in loss of aggrecans from cartilage likely due to reduced intra-cartilage
osmotic swelling pressures [15]. The high dosage of Avidin at a concentration of 100 μM has been shown to induce 2-folds greater GAG loss from cartilage compared to untreated control, however, a dose of <10μM didn’t cause any GAG loss from bovine cartilage explant [20]. Therefore, in order to effectively use the cartilage targeting property of Avidin for delivering drugs for OA treatment, it is necessary to re-design the carrier such that 10x lower amount of Avidin can be used for delivering the same amount of Dex.

With the goal of increasing the drug loading content on Avidin, the objectives of this study were to (1) create a multi-arm Avidin (mAv) structure by conjugating Avidin with four 8-arm-PEGs thereby providing larger number of sites for Dex conjugation, (2) characterize cartilage transport properties of multi-arm Avidin, and (3) conjugate Dex to multi-arm Avidin using hydrolysable ester linkers and characterize drug release rates.
2. Materials and Methods

2.1 Materials

10 kDa 8-arm Polyethylene glycol (PEG) amine hydrochloride salt was purchased from Advanced Biochemicals (Lawrenceville, GA). N-Hydroxysuccinimido (NHS)-Biotin, 1-ethyl-3-(3 dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysulfosuccinimide (NHSS), Avidin and Avidin-Texas red conjugated (Avidin-TR), 4’-hydroxybenzene-2-carboxylic acid (HABA), 7 kDa MWCO SnakeSkin dialysis tubing was purchased from Thermo Fisher Scientific (Waltham, MA). Dexamethasone (Dex), Succinic anhydride (SA), Pyridine, fluorescein isothiocyanate isomer I (FITC), Sinapic acid matrix (SA matrix) and other salts were purchased from Sigma-Aldrich (St. Louis, MO).

2.2 Chemical synthesis

2.2.1 Biotinylation of 8-arm PEG

PEG was biotinylated by reaction with NHS-Biotin. Briefly, 10 mg of PEG was dissolved in 500 μL of deionized water (DI) water and 1.67 mg of NHS-Biotin was dissolved in 700 μL of Dimethyl sulfoxide (DMSO), as shown in Fig 1. NHS-Biotin solution was then added dropwise to the PEG solution (5:1 molar ratio) and reacted for 1 h under gentle rotation at room temperature. Excess NHS-Biotin was removed from the PEG-Biotin conjugate solution using dialysis (7 kDa MWCO) for
24 h against PBS buffer. Extent of biotinylation was confirmed using MALDI-TOF and the HABA dye assay.

2.2.2 Loading of PEG-Biotin on Avidin to synthesize multi-arm Avidin (mAv)

PEG-Biotin from section 2.2.1 were reacted with Avidin in DI water for 30 minutes under gentle shaking at room temperature (as shown in Fig 1) in 1:1, 2:1, 3:1, 4:1 through 8:1 molar ratios to determine the ratio at which all four biotin binding sites of Avidin are occupied with PEG-Biotin, which was confirmed using the HABA dye assay, gel electrophoresis and UPLC. The product was then freeze-dried and stored at -20 °C until further use.

Figure 1 Schematic of mAv chemical synthesis.
2.2.3 Synthesis of Dexamethasone hemesuccinate (Dex-SA)

Carboxylated derivatives of Dex were prepared by reacting 36 mg Dex (0.092 mmol, 1.0 equiv) with 46 mg of succinic anhydride (SA), in presence of 2 mg Dimethylaminopyridine (DMAP) (0.0152 mmol, 0.1 equiv) as a catalyst in 1 mL of pyridine. The reaction for Dex-SA was conducted in a round bottom flask purged with nitrogen gas for 24 h at room temperature. Following completion of the reaction, pyridine was evaporated with constant purging of nitrogen gas, and 4 mL of the cold solution containing 25 mL water and 10 mL HCl was added to the flask to precipitate Dex-SA. A white precipitate was observed, which was stirred for 10 minutes and then centrifuged at 10,000 g for 5 min for 5 cycles. In each cycle, the supernatant was replaced with fresh cold solution. The final product of Dex-SA was lyophilized, weighed and stored at -20 °C for future use. Their structures were confirmed using ¹H-NMR.

2.2.4 Conjugation of Dex-SA to PEG-Biotin

Dex-SA was conjugated to PEG-Biotin using EDC-NHS chemistry. Briefly, 5 mg of Dex-SA (0.01 mmol, 100 equiv) was dissolved initially in 120 μL of DMSO and added 600 μL of 2-morpholinoethanesulfonic acid (MES) dropwise. Then, 19.2 mg of EDC (0.104 mmol, 1040 equiv) and 21.7 mg NHSS (0.092 mmol, 92 equiv) was added to Dex-SA solution, and all of them were purged with nitrogen to activate the reaction for 30 minutes. Subsequently, 1 mg of PEG-Biotin (0.1 μmol, 1 equiv) was added to each of the solution and reacted for 2 h at room temperature, purging the nitrogen as well. Upon completion of the reaction, the final product was dialyzed using 7 kDa MWCO membrane to remove the excessive reagents under
4 °C for 24 hours. The pure product was then lyophilized and stored at -20 °C for future purposes.

2.3 Analysis

2.3.1 MALDI-TOF spectrometry

10 μL of 8-arm-PEG (1mg/ml), and 10 μL of PEG-Biotin (1mg/ml) was mixed with 10 μL sinapinic acid (SA) matrix separately to be tested by the MALDI-TOF spectrometry (Bruker Microflex II) to confirm the numbers of biotin per PEG.

2.3.2 HABA colorimetric assay

The extent of biotinylation in PEG-Biotin and the loading of PEG-Biotin on Avidin were determined by using the HABA colorimetric assay [21]. Changes in absorbance of HABA-Avidin complex at 500 nm due to competitive displacement on biotin sites of Avidin with biotinylated PEG was used to estimate the degree of biotinylation. Briefly, HABA dye was dissolved in 10 mL of nanopure water (2.42 mg/mL) and filtered using 0.2 μm filter. Excessive HABA dye was added to Avidin solution to a final concentration of 0.82 mg/mL (initial absorbance of 1.2). 20 μL of graded concentrations of PEG-Biotin was added to 180 μL of HABA-Avidin complex (1:1 through 8:1 molar ratio of PEG-Biotin to HABA-Avidin) that competitively displaced HABA from Biotin binding sites of Avidin thereby reducing the absorbance value. 100% biotinylation of Avidin was achieved when the change in absorbance achieved a plateau. This was also confirmed by calculations using methods from the standard Beer Lambert Law [21].
2.3.3 Gel electrophoresis

Conjugation of PEG-Biotin to Avidin was confirmed by using native PAGE in 7.5% separating gel. In brief, 12 μL of protein samples (~7.5 μg protein) were mixed with 4 μL of 4x Laemmli sample loading buffer (Bio-RAD) without any heating. Because the isoelectric point of Avidin is 10.5 and since the protein mobility depends on both the charge and molecular weight in the native PAGE gel, the electrode polarity had to be reversed (anode was inserted at the top of gel and cathode was inserted at the bottom of gel). Electrophoresis was performed for approximate 4 h in 1x solution of non-SDS tris-glycine running buffer at 200 V, 40 mA and 4 °C.

Native gel was stained using iodine and Coomassie Brilliant Blue R-250. Briefly, gel was fixed and then washed with DI water for 20 minutes, then incubated in 5% barium chloride solution for 15 minutes followed by 3 washes in DI water. Subsequently, the gel was stained with potassium iodide and iodine solution for 5 minutes to identify free or conjugated PEG. Following this, the gel was stained with Coomassie Brilliant Blue R-250 for Avidin, and destained three times in 100 mL of Distaining solution (50% water, 40% Methanol, and 10% acetic acid) for overnight.

2.3.4 Ultra-High-Pressure Liquid Chromatography (UPLC) and Zeta Potential

Pegylation of Avidin was further confirmed by using an H-Class Acquity UPLC (Waters Corp, Milford, MA) equipped with an Acquity UPLC BEH200 Size Exclusion Column (200Å, 1.7 μm column, 4.6 x 300 mm) with 20 mM ammonium bicarbonate buffer as the mobile phase at 0.2mL/min. Avidin was detected at 280
nm. Zeta potential of Avidin and pegylated Avidin was measured in nanopure water at 0.45 mg/mL concentration using a Zetasizer Nano-ZS90.

2.3.5 Proton Nuclear Magnetic Resonance (¹H-NMR)

Modification of Dex and conjugation of Dex-SA to PEG-Botin were verified using 500 MHz ¹H-NMR (Varian Inova. Agilent Technologies). 1-2 mg of mixture of PEG and Dex-SA, and PEG-Dex-SA conjugation were dissolved in 700 μL DMSO-d6. The obtained NMR data was analyzed using MestRe Nova software.

2.3.6 Dex loading content

Dex-PEG-Biotin was hydrolyzed using 0.1 N hydrochloric acid overnight and neutralized against 0.1 N sodium hydroxide. The amount of Dex released was quantified by HPLC (Agilent Technologies 1260 infinity II) equipped with a UV detector. Poroshell 120 EC-C18 4.6x150 mm column was used. A gradient of solvent A (0.1% TFA in water) and solvent B (0.1% TFA in acetonitrile) was used. The concentration of solvent B was increased linearly from 5% to 65% over 15 min. Column temperature of 30 °C and a flow rate of 1.0 mL/min were used. Dex was detected at 254 nm. Data were interpreted with the UNIFI software (Waters Corp, version 1.7.1). Drug loading content (DLC) was calculated as number of 26.4 Dex moles per mole of Avidin.

2.3.7 In-vitro Dex release rates

Dex release rates from free Dex and Dex-PEG-Biotin were estimated in PBS at pH 7.4, 37 °C using dialysis tubing (7 kDa MWCO) with continuous shaking under non-sink conditions. At different time intervals, 200 μL of release media was
used to estimate the Dex concentration by HPLC, which was replaced by equal amount of fresh release medium.

2.4 Transport studies

2.4.1 Equilibrium uptake in cartilage and intra-cartilage retention.

Cartilage explants were harvested from the femoral patellar groove of 2-3-week-old bovine knees (Research 87, Boylston, MA) with a 3 mm diameter biopsy punch. The cylindrical plugs were then sliced to obtain the superficial 1 mm layer of cartilage, and frozen until use. Avidin was labeled with Texas red and conjugated with FITC labeled 8-arm PEGs. Cartilage disks were equilibrated in 300 μL of 8.5 μM of labeled Avidin, mAv 1:2 and mAv 1:4 in presence of protease inhibitors for 24 h in a 96 well plate at 37 °C (as shown in Fig 2). After 24h of equilibration in Avidin or mAv bath, explants were transferred into 1x or 10x PBS for 7 days. The bath solution was changed into fresh 1x or 10x PBS every day. Then the fluorescence of solution was measured with plate reader. Percentage retention inside of cartilage was calculated by normalizing the total Avidin or mAv moles remained inside the explant after desorption by the total initial Avidin or mAv uptake by the tissue after 24 h [22].
The equilibrium bath fluorescence was measured using a plate reader. The final concentration was calculated based on a linear calibration correlating fluorescence to concentration of labeled Avidin. The moles of solutes absorbed into the cartilage were calculated using the difference between the initial and equilibrium concentration of the bath. The concentration of solutes inside was calculated by normalizing the number of moles inside cartilage to the wet weight of the tissue. The uptake ratio ($R_U$) was defined as the ratio of the concentration of solutes inside the tissue ($C_{tissue}$) to that of the solute in the equilibrium bath ($C_{bath}$).

$$R_U = \frac{C_{tissue}}{C_{bath}}$$

### 2.4.2 Confocal imaging of depth of penetration into cartilage

A previously described transport setup was used to study 1-dimensional diffusion of solutes in cartilage [22]. Briefly, 6 mm half disk cartilage explants were mounted in the mid-section of the chamber. The upstream compartment was filled with either 9.5 μM of Avidin-Texas red, dual labeled mAv 1:2 or mAv 1:4. The transport chamber was placed in a petri dish containing water to minimize evaporation and placed on a shaker inside the incubator (as shown in Fig 3). After
24 h of adsorption, a 100 μm slice was cut from the center of the explant and imaged using a confocal microscope (Zeiss LSM 700). Texas red was excited using 555 nm laser line and FITC was separately excited using 488 nm laser line. Z-stack multilayer image of both channels (Red and Green) were obtained to visualize distribution of mA v conjugates. The maximum intensity of each channel was projected to the z-axis.

**Figure 3** Diffusion transport chamber setup for confocal microscopy of tissue.
3. Results

3.1 Characteristics of multi-arm (mA\textit{v})

Biotinylation was confirmed using MALDI-TOF mass spectrometry firstly, molecular weight of PEG-biotin was 10902 Da and 8-arm-PEG was 10620 Da (as shown in \textbf{Fig 4}). Since molecular weight of biotin was 245 Da, so difference between them showed that 1.15 biotin was labeled with per Avidin. Besides, the average number of Biotins on per PEG was $1.28 \pm 0.02$ which was determined with Beer Lambert Law\cite{21}, based on the absorbance drop tested using HABA assay, which was consistent with the mass spec data. In addition, titration curve of PEG-Biotin showed the reduction of absorbance with increasing molar ratio of Biotinylated PEG to Avidin from 1:1 to 4:1, following which a plateau was achieved meaning that all the Biotin sites on Avidin were occupied with PEG-Biotin indicating the formation of mA\textit{v} (\textbf{Fig 5}).

\textbf{Figure 4} MALDI-TOF spectrometry of PEG and PEG-Biotin (molar ratio 1:1).
Synthesis of multi-arm Avidin (mAv) was confirmed using native PAGE gel electrophoresis as well. Native PAGE gel electrophoresis confirmed that Avidin was PEGylated; channels in Fig 6 showed Avidin only, PEG only, mAv 1:2 (molar ratio of avidin to peg is 1:2) and mAv 1:4 (molar ratio of avdin to peg is 1:4 ), with two different staining methods, PEG staining with iodine and protein staining with Coomassie brilliant blue. In PEG-staining (left), bands only appeared in PEG channel and mAv channel. However, in protein-staining (right), bands showed in Avidin and mAv channels but not in PEG, as expected. Therefore, the same position in mAv channel in both PEG staining and protein staining verified that Avidin was successfully PEGylated Avidin. Additionally, mAv band is smudged compare to Avidin only and it covered shorter distance in the channel compared to avidin’s band due to its increased molecular weight (as shown in Fig 6).
Figure 6 Native-PAGE Gel of Avidin, mAv, mAv 1:2 and mAv 1:4 with reversed polarity with PEG staining (left) and Coomassie brilliant blue staining (right).

Size exclusion column was used to further confirm the structure mAv (as shown in Fig 7). Avidin as a control group showed that the retention time is 6.29 minutes, for mAv 1:2 is 5.33 minutes and mAv 1:4 is 4.38 minutes. The ratio of mAv 1:4 in this formulation is 60% (as shown in Fig 7). While the diameter, shown in Table 1, for each of formulation was derived from Erickson Function [23]. A slightly increase of the size for mAv 1:2 and mAv 1:4 is due to the molecular weight change caused by the numbers of PEG labeled with Avidin. Additionally, net Charge for Avidin, mAv 1:2, and mAv 1:4 were measured and shown in the Table 1, indicating that 8-arm-PEG doesn’t shield the net charge of Avidin. However, the slightly charge increase in both mAv 1:2 and mAv 1:4 is because of the free amine group in the PEG provided the positive charge when the mAv dissolved in nanopure water.
Figure 7 Avidin and mAv 1:4 dissolved in nanopore water for UPLC analysis with size exclusion column. Peak for Avidin is at 6.29 (left); two main peaks for mAv 1:4 are shown. Peak for mAv 1:4 is at 4.38; peak for mAv 1:2 is at 5.33 (right).

Table 1. Net size, charge and intra-cartilage transport properties of Avidin, mAv 1:2 and mAv 1:4.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Avidin</th>
<th>mAv 1:2</th>
<th>mAv 1:4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter (nm)</td>
<td>~7</td>
<td>~7.6</td>
<td>~8.1</td>
</tr>
<tr>
<td>ζ (mV)</td>
<td>18.3 ± 0.5</td>
<td>20.3 ± 0.3</td>
<td>25.3 ± 0.7</td>
</tr>
</tbody>
</table>

3.2 Transport of mAv conjugates in bovine cartilage

Equilibrium uptake ratios (R_U) of mAv conjugates and Avidin are shown in Fig 8. Both mAv conjugates showed lower R_U in cartilage compared to Avidin due to the size of mAv increase. However, mAv conjugates still maintained high uptake in cartilage (R_U of 96 and 112 for mAv 1:2 and mAv 1:4, respectively). Additionally, mAv conjugates maintained similar percentage retention inside of cartilage in 1x PBS compare to Avidin for 7 days. 10x PBS was able to shield charge interaction. Therefore, all of them released out from the inside of cartilage indicating that
charge played an important role in percentage retention inside of cartilage of Avidin and mAv (as shown in Fig 9).

**Figure 8** Intra-cartilage equilibrium uptake of Avidin, mAv 1:2 and mAv 1:4 after 24 h.

**Figure 9** Avidin, mAv 1:2 and mAv 1:4 percentage retention in cartilage following desorption in 1x PBS and 10x PBS over 7 days.
Depth of penetration of mA 1:4 in cartilage is shown in Fig 10. mA 1:4 was able to penetrate through the full thickness of cartilage within 24 h, similar to Avidin [15]. The green channel shows presence of PEG-FITC while the red channel shows Avidin-Texas red distribution in cartilage (as shown in Fig 10).

Figure 10 Confocal microscopy images demonstrating full thickness intra-cartilage penetration of mA 1:4 from superficial zone (SZ) to deep zone (DZ) of cartilage. Red channel shows Avidin-TX and green channel shows presence of PEG-FITC.

3.3 Characteristics of PEG-Dex

3.3.1 Confirmation of PEG-Dex

Dex-SA was successfully synthesized through the esterification between hydroxyl group in DEX and carboxyl group from anhydride of SA with the DMAP as a catalyst. Incorporation of carboxyl group in the Dex-SA was verified by TLC plates (data not shown). In addition, the amide bond has been used to link the amine group in PEG and the carboxyl group in Dex-SA through the EDC-NHS reaction, then verified by NMR to confirm the structure of PEG-Dex-SA. Taking the mixture of PEG and DEX-SA as a negative control, the proton peak of PEG (δ = 3-
3.5); olefin in Dex ($\delta = 6-6.5$), showed in both of the samples. Amino, however, in PEG-Dex-SA ($\delta = 8$) was only observed in conjugation PEG-Dex-SA in $^1$H-NMR spectrum indicating that the reaction happened (as shown in Fig 11).

![Figure 11 $^1$H NMR spectrum of PEG and Dex-SA mixture and PEG-Dex-SA, conjugation.](image)

3.3.2 PEG-Dex in-vitro drug loading content and drug release study.

HPLC showed the retention time for Dex was at 12.255 minutes (254 nm). Based on the peak in HPLC, with hydrolysis to break the ester bond between Dex and PEG, the moles of Dex, detected by the HPLC, per PEG was $6.64 \pm 0.5$. While release profile of free Dex in PBS at pH 7.4 and 37 °C was shown in Fig.12. 80% of Dex released from free Dex condition after 2 h. However, Dex conjugated with
PEG with ester linker resulted in the slower release rates compared to free Dex and the half-life of it was $6.8 \pm 0.2$ h (as shown in the Fig 12).

![Graph showing drug release profiles](image)

**Figure 12** In vitro drug release profiles of free Dex and PEG-Dex-SA conjugation in pH 7.4 PBS at 37 °C.
4. Discussion

Glucocorticoids (GCs) have been evaluated for treating OA disease over 50 years [24]. Most studies have reported these drugs to be safe and efficient for pain relief and increasing the motion following treatment [25, 26]. More importantly, GCs which are able to significantly suppress the inflammatory cytokines TNFα, TNFα+IL-6, and IL-6 have been wildly used for treatment of inflammatory conditions [6, 27, 28]. However, the efficacy of glucocorticoids depended on the dosage, frequency and duration of administration [29]. Recent, it was reported that in PTOA rabbit model, high dosage (0.5 mg/kg of rabbit body weight) and repeated administration of Dex caused body loss and adverse systemic effects on several organs of the tested animals [8]. However, when a single dose of high dosage of Dex (0.5 mg/kg of rabbit body weight) was injected into the PTOA rabbit model, no adverse side effects were observed, but cartilage only appeared to be partially protected [30]. Therefore, it is crucial to modify the drugs to treat the PTOA disease with sustained low drug doses over a long period of time, avoid the adverse side effects and at the mean time enhance the drug efficiency [31].

In order to decrease the side effects following by the frequent injections or administration of high drug concentrations, such as bone desorption, cell apoptosis and systemic toxicity after IA injection [32, 33], it is important to develop strategies to help the drug to penetrate into the middle and deep zone of cartilage where the majority of chondrocytes are located before cleared out from the joint space [14, 17]. However, most of drug delivery systems cannot penetrate into the cartilage,
leading to an ineffective treatment in OA disease. For example, micron sized polymeric particles such as PLGA microsphere containing the TCA was only able to retain in the synovial fluid over the period of the treatment [34]. Hydrogels encapsulating the Dex failed to penetrate into the cartilage as well [35]. However, recently, cationic drug delivery systems have been developed to penetrate the full thickness of cartilage, rather than creating an intra-joint drug depot system only [17-19]. This way, using the negative fixed charge density of cartilage, cartilage ECM is converted from an avascular barrier to drugs into a drug depot system via charged interactions [36]. Avidin, due to its size (~7 nm) and high positive charge, has shown to be applied as a drug delivery system with fast diffusion rate in cartilage taking advantage of weak-reversible charge interactions with the negatively charged GAGs [15]. Besides, the binding interactions, between the Avidin and negative tissue proteoglycans inside of the cartilage, enabled Avidin to behave like a drug depot in the deep zone of the cartilage to achieve a sustainable release [15]. mAv, due to the its 8 nm size and +22 mV net charge, containing high intra-cartilage uptake (Ru~110) and similar high intra-cartilage retention as avidin, is able to convert the cartilage from a drug diffusion barrier into a drug depot.

The objective of this study is to establish a drug delivery system which enables more (drug) Dex with less (carrier) avidin to penetrate into the full thickness of cartilage. The drug delivery system behaves as a drug depot that allowed sustained release of Dex to the chondrocytes which suppressed cytokine induced GAG loss and enhanced chondrocyte viability. Previously it was reported that single dose delivery of Avidin-Dex conjugate comprised of 4 moles of Dex per
Avidin significantly suppressed GAG loss and prevented the chondrocyte death induced by the inflammatory cytokines [18]. Additionally, this drug delivery system showed promising suppression of inflammation after injection into a PTOA rabbit model. However, using higher concentration of Avidin (300 μM) in this system reduced the osmotic pressure of cartilage because of its cationic charge, inducing loss of water content and GAG [19]. In this study, we improved the drug delivery system by incorporation of an 8-arm PEG that provides 28 sites for Dex conjugation per Avidin compared to only 4 sites as mentioned before. Therefore, a higher drug loading efficacy for this drug delivery system enabled using lower concentration of Avidin as the carrier.

Although here we successfully synthesized mAv-Dex with high drug loading content, faster drug release rates of ester linker, drug half-life is only 6.8h in this study, which will lead to significant drug loss in the media before diffusion of mAv-Dex conjugates into the cartilage. The fast hydrolysis rate of the ester bond in mAv-Dex might occur due to presence of the adjacent carbonyl group in the amide bond attempted to compete and withdraw electrons from ester linker which weak the bond stability [37]. Furthermore, hydrophobic Dex conjugated to hydrophilic polymer PEG will produce mutual exclusive force between each other which accelerated ester bond hydrolysis [38]. Therefore, it’s important to increase the distance between the Dex and PEG by adding more carbon between the ester bond and amide bond. The concept has been approved by using the Diethyl Methoxy phthalates which contain an aromatic nucleus to increase the half-life of ester linker [39]. Additionally, in in-vivo experiments only 35%-40% of IA injected
Avidin was retained inside of the joint after 24 hours [40] indicating that more than 60% of drug won’t be able to be delivered into the middle zone and deep zone of cartilage. Together, a longer half-life linker is necessary by increasing the carbon length between the amide bond and ester bond not only for improving the treatment efficiency, but also for reducing the side effect induced by drug or carrier.

Pro-anabolic drugs are a different category of agents which have shown successful suppression of cytokines in vitro [7, 41, 42]. Our system is capable of delivery of this class of drugs through similar conjugation chemistry as described here. Kartogenin (KGN), a pro-anabolic drug has been shown to reduce GAG loss to 60% GAG loss in a TNFα induced cytokine model, following treatment with 5 μM KGN compared to TNFα only condition [41]. Similar result about the suppression of inflammatory cytokines with KGN reported that when treating KGN with IL-1β, it can reduce GAG release from bovine articular cartilage explant significantly compared to IL-1β treatment alone [42]. More importantly, the combined treatment of anti-catabolic drug (Dex) and pro-anabolic drug (IGF-1) was reported to be more efficient in suppressing the inflammatory cytokines in the cartilage than treating it only with either of the cases [7]. Therefore, this way, our drug delivery system, mAvt, is able to reach a comprehensive treatment in OA disease through delivery of both types of the drug.
5. Conclusion

Electrostatic interactions can be applied to improve the targeted drug delivery to negatively charged tissue like cartilage. Besides, negatively charged tissue was converted from a barrier to drug entry into a drug depot using positively charged protein, Avidin, as a drug carrier. Therefore, Avidin creates an intra-cartilage drug depot offering sustained drug release over a long time period [20, 22]. Here we improved Avidin drug loading by increasing the number of drug binding sites through addition of 4 multi-arm PEG. By introducing 28 free conjugate sites in the mAv for drug conjugation through amide bond, 7 times less concentration of Avidin comparing with previous research [19] can be utilized for in-vitro and in-vivo in the future. This way, an effective concentration of Dex can reach the target sites located in deep zone of cartilage for a more competent and longer period treatment.
6. Future Direction

In the next stage, we will firstly test the treatment efficacy of mAv-Dex comparing to single dose Dex and continuous dose Dex in in-vitro cytokine challenged PTOA model by treating IL-1 to the bovine cartilage explant mentioned in the study. Secondly, in order to reach the sustainable release, the linker between PEG-amine to Dex is key to control the release rates by increasing the carbon length. Last but not least, it is promising that conjugate both pro-anabolic drugs (KGN) and anti-catabolic drugs (Dex) to the Multi-arm mAv to reach to the combination of therapeutic treatment of OA.
7. REFERENCES


