CHARGE-BASED TRANSPORT AND DRUG DELIVERY INTO CARTILAGE FOR LOCALIZED TREATMENT OF DEGENERATIVE JOINT DISEASES

by

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Submitted to the Department of Mechanical Engineering
In Partial Fulfillment of the Requirements of the Degree of Doctor of Philosophy in Mechanical Engineering

At the

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Abstract

Traumatic joint injuries significantly increase synovial fluid levels of pro-inflammatory cytokines that can initiate cartilage degeneration leading to osteoarthritis (OA). Articular cartilage is a highly negatively charged, avascular tissue, which relies on synovial fluid convection and electro-diffusion to transport proteins and therapeutics to tissue chondrocytes. No OA drug has yet passed the safety criteria of clinical trials due to ineffective intra-articular (i.a.) delivery methods, which require very high drug doses that cause systemic toxicity. There is a need to design local delivery mechanisms that can enable drugs or drug carriers to rapidly diffuse into the cartilage extracellular matrix to achieve intratissue therapeutic levels before these drugs are cleared from the joint space via lymphatics and synovium vasculature.

This dissertation investigates the effects of size and charge of solutes on their penetration, binding and retention within negatively charged tissues such as cartilage. Based on this understanding we selected Avidin, a globular protein, as a drug carrier owing to its optimal size and high positive charge (66,000 Da, pl 10.5). Avidin resulted in a six-fold upward Donnan partitioning factor at the synovial fluid-cartilage interface, had a 400-fold higher uptake than its electrically neutral counterpart (Neutravidin), and remained bound within cartilage for at least 15 days. Competitive binding experiments revealed that despite Avidin’s weak and reversible ionic binding (dissociation constant, \(K_D \sim 150 \mu M\)) to the negatively charged glycosaminoglycans, its long term retention was facilitated by large intratissue binding site density (\(N_T \sim 2,920 \mu M\)). Thus, structures like Avidin are ideal candidates for local i.a. drug delivery into cartilage.

In vivo animal studies revealed that Avidin retained inside the joint space for extended time periods resulting half-life of 154h in rabbit cartilage which was 5-6 times longer than that in the thinner rat cartilage. This was confirmed to be consistent with the concept that diffusion-binding kinetics scale as the square of tissue thickness, emphasizing the necessity of using larger animal models for studying joint space transport and pharmacokinetics. Avidin’s neutral counterpart (Neutravidin) was completely cleared from the joint space of both rats and rabbits within 24h.

We then conjugated Avidin with the glucocorticoid, dexamethasone, using chemical linkers to enable its sustained release. Avidin delivered dexamethasone into cartilage deep zones where majority of chondrocytes reside thereby successfully inhibiting cytokine-induced catabolic activity in cartilage explants in-vitro. A single i.a. injection of Avidin-conjugated drug can thereby enable sustained drug delivery in low doses and therefore has the potential to replace the current clinical practice of using
multiple injections of high dose glucocorticoids in patients. The biological efficacy of this system in rescuing degenerative mechanisms of OA is currently being validated in a well-accepted rabbit model of post-traumatic OA as part of a preclinical study.

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To my teachers
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-Ambika Goel Bajpayee

-अम्बिका गोएल बाजपायी
Lead me from the unreal to the real;
from darkness (ignorance) to light (knowledge);
and from death to immortality.

Bṛhadāraṇyaka Upaniṣad
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Chapter 1    Introduction

1.1. Osteoarthritis, a degenerative joint disease

Osteoarthritis (OA) is a complex debilitating disease that affects millions of people worldwide, causing loss of productivity, quality of life, and loss of joint function. OA affects over 150 million people worldwide, about 25 million people in the US and this number is expected to double by 2020 (Source: Disease, Incidence, Prevalence & Disability; WHO 2009, data based on census in 2004) making it the fourth leading cause of disability by 2020. Yet, there is no cure for it.

![Prevalence of Osteoarthritis](image)

![Life years lost due to Osteoarthritis](image)

It is now accepted that OA is a disease of the entire joint, eventually affecting all joints tissues including cartilage, bone, ligaments, menisci, the joint capsule, synovial membrane, muscles and neural tissue [1,2]. Distinct subtypes of OA are associated with the varying risk factors that mediate OA initiation and progression; these risk factors include improper joint mechanics, gender, age, obesity, genetic and metabolic factors, and acute joint injury leading to post-traumatic OA (PTOA) [3, 4]. PTOA accounts for 12% of the total OA population [5]. Approximately 50-80% of young active individuals who
suffer traumatic joint injuries (e.g., rupture of the anterior cruciate ligament or meniscus) progress to PTOA within 10-20 years [6, 7]. Following acute joint injury, there is an immediate increase in synovial fluid levels of inflammatory cytokines (e.g., IL-1, IL-6, TNFα) which can diffuse into cartilage and rapidly initiate proteolysis and loss of cartilage matrix [8, 9]. By the time of clinical (radiographic) diagnosis, irreversible changes to cartilage and other joint tissues have often occurred [6]. Since the cause and time of the initial trauma is known, there exists a unique opportunity for early drug intervention to prevent further degeneration of cartilage and other tissues, and to reverse the course of PTOA by inducing repair [6].

While there are disease-modifying anti-rheumatic drugs (DMARDS) for rheumatoid arthritis and several related rheumatic diseases (such as the TNFα-blockers [10], Table 1.1), no efficacious disease-modifying osteoarthritis drugs (DMOADs) are yet available, i.e., drugs which alter or halt the progression of OA [1,11,12]. Current therapies provide only short term relief of pain and inflammation (e.g., analgesics, hyaluronic acid lubricants, etc.), but afford no protection against further degeneration of cartilage, the hallmark of end-stage OA [3], leading to the need for joint replacement. Several anti-catabolic and pro-anabolic drugs have been identified as potentially useful to reverse or prevent PTOA-associated breakdown of cartilage, including anti-catabolic glucocorticoids (e.g., dexamethasone) and pro-anabolic growth factors (e.g., IGF-1, FGF-18, and BMP-7) [11,13-15]. Consistent with the concept that generalized OA involves the whole joint, DMOAD development and associated clinical trials are now targeting cartilage breakdown (e.g., protease and cytokine inhibitors), bone remodeling (e.g., bisphosphonates, BMP-7, calcitonin), and synovial and inflammatory mediators (e.g., cytokine blockers) [11]. Table 1.1 provides a list of therapeutics currently being considered for OA treatment delivered via intra-articular injections. Biological agents such as monoclonal antibodies for inhibiting IL-1β (Canakinumab), TNFα (Infliximab, Adalimumab) and other anti IL-1 or TNFα agents (such as, Anakinra and Enbrel) have been successfully used for systemic treatments for rheumatoid arthritis and are being considered for i.a. therapy for OA treatment. However, no drug candidates have yet passed the safety/efficacy hurdle, and systemic drug side-effects have been a major safety concern causing several trial failures to-date [12]. Anakinra (IL-1Ra) showed only short term benefit in its phase II evaluation as an i.a. treatment for OA owing to rapid clearance from the joint due to its small size (17kDa). Thus, it is important to develop appropriate drug delivery methods to administer potentially efficacious drugs or drug combinations directly to selected target tissues, such as cartilage thereby eliminating any systemic adverse effects [16].
1.2. Intra-articular (i.a.) therapy and the need for intra-tissue drug delivery

Intra-articular injection has enabled local administration of drugs into the joint space and thus reduced systemic toxicity and improved drug bioavailability; yet it remains inadequate due to rapid exit of drugs from the joint space. Small molecules exit via the rich synovial capillary network while the larger macromolecules (e.g., hyaluronan) are cleared by the lymphatic system, which is located in the subsynovium [17, 18]. Short mean half-lives of NSAIDs (Non-Steroidal Anti-Inflammatory Drugs) in the synovial fluid have been reported as 1-4 h [19, 20]. Furthermore, simple i.a. injection can require very high drug doses to achieve a concentration gradient high enough for drug diffusion into cartilage. Such high doses can cause deleterious effects; even low-dose sustained release in the synovial fluid may still cause unwanted drug exposure to other joint tissues.

It is important to note that most therapeutics listed in Table 1.1 (except hyaluronan and many proteinase inhibitors) such as glucocorticoids, and growth factors have to be delivered to the chondrocytes, most of which reside in the deep zones of cartilage. Fig. 1.1 shows example mechanisms of how therapeutics interact with their intracellular or extracellular target binding sites to inhibit catabolic effects and restore normal biosynthesis levels. High molecular weight, hyaluronan (clinically approved, e.g., Sanofi’s Synvisc is 6000 kDa in MW) or lubricin-like products restore properties of synovial fluid in the joint space and make a film about 1 mm thick on cartilage surfaces. Thus their target sites are in the superficial surfaces of cartilage, synovial membrane and in the synovial fluid. Synovial fluid is essential for joint lubrication and mechanical sliding that enables diffusion of nutrients into the avascular cartilage.

New approaches have focused on delivery of functionalized drugs directly into the joint space-synovial fluid using micro and nanoparticles [21-25], drug releasing peptides and gels [26-28]. Most of these systems are aimed at increasing the residence time of drugs in synovial fluid but are unable to transport drug/drug carriers directly into the deep zones of cartilage where the majority cell/tissue targets reside. Entry of macromolecules into cartilage is hindered by its complex architecture (discussed in section 1.3) of dense collagen fibrils and negatively charged aggrecan proteoglycans. Thus, cartilage is often considered as a ‘barrier to drug/drug carrier entry’.

1.3. Structure of cartilage

Articular cartilage is a highly complex, avascular, alymphatic and aneural tissue made of a dense network of collagen fibrils (constituting 50-60% dry weight of tissue), aggrecan proteoglycans that contain highly negatively charged glycosaminoglycan (GAG) chains (30-35% tissue dry weight) and
many additional extracellular proteins which are continuously synthesized by a low density of chondrocytes (1-5% tissue dry weight). Type II collagen is the most abundant type present and its mesh pore size is 60-200 nm. The superficial zone (SZ) of cartilage (the most superficial and well lubricated region that provides gliding surface and is about 10-20% of total cartilage thickness) has collagen fibrils aligned parallel to the surface. The collagen fibrils are oriented randomly in the middle zone (MZ, 40-60% tissue thickness) and are perpendicular to the subchondral bone in the deep zone (DZ, 30-40% tissue thickness). The calcified zone provides a mechanical transition between the soft cartilage tissue and the stiff subchondral bone. The collagen network is space-filled with 300MDa aggregates of aggrecan, having sulfated GAG chains that are only 2-3 nm apart from each other. The aggrecan aggregates have a bottle brush like structure comprised of a long central hyaluronan (HA) GAG chain that links electrostatically to 3MDa aggrecan monomers at their G1 binding domains. This binding is further stabilized by link protein (Fig. 1.2), which has a structure analogous to that of the aggrecan G1 domain.

The aggrecan and chondrocyte densities increase with depth into cartilage from the superficial zone, which further reduces the pore size and restricts the ability of solutes to penetrate and diffuse within cartilage. There is a lack of understanding of what size range of solutes can penetrate through full thickness of dense cartilage ECM (extracellular matrix), and this will be explored further in Chapter 2.

In summary, there are two competing rates of transport inside the joint space for drug carrying particles: (i) the lumped rate of transport of particles into soft tissues such as cartilage (i.e., the diffusive flux of entry into the soft tissues, \( Q_{\text{Entry}} \)) and (ii) rate of exit from the lymphatics (the diffusive flux of exit from the joint space, \( Q_{\text{Exit}} \)). \( Q_{\text{Entry}} \) should be such that it can enable fast enough transport of drug carriers inside the desired target tissue to achieve intra-tissue therapeutic levels before the majority of the drug carriers are cleared from the joint space (\( Q_{\text{Exit}} \)). Solute flux (\( Q \)) in a given medium is related to the concentration (\( C_i \)) gradient and its diffusivity by Fick’s first law of diffusion:

\[
Q_i = -D_i \nabla C_i + C_i v ,
\]

where \( D_i \) is the effective solute diffusivity in the medium and \( v \) is the synovial fluid velocity. Joint space kinetics are further explored in Chapter 3.

1.4. Charge based mechanisms for local intra-tissue drug delivery

The high density of heavily negatively charged GAGs inside cartilage provides a unique opportunity to use electrostatic interactions to augment transport rate, uptake and binding of drug carriers
inside the tissue. In fact, the negatively charged aggregan proteoglycans are also present in other soft
tissues such as menisci, ligaments and in small concentrations in tendons (See Chapters 4 and 5). The
synovial fluid (SF) also has a high density of the negatively charged hyaluronic acid (HA) and lubricin.
The viscosity of SF is attributed to these two molecules, which provide effective hydrodynamic
lubrication necessary for healthy functioning of joints. SF forms a thin layer on cartilage surfaces,
synovial membrane and fat pads, providing retention pockets for cationic drug carriers. The infrapatellar
fat pad (Hoffa) is the largest fat pad in the joint and also contains sulfated GAGs as well as non-sulfated
HA [29].

The high fixed charge density (FCD) within the ECM of such tissues provides a natural reservoir
for cationic drug carriers, yet this attribute of joint tissues has not been fully exploited for local i.a. drug
delivery. This aspect, however, has been effectively used in physiological imaging techniques such as
delayed gadolinium enhanced medical resonance (MR) imaging of cartilage (dGEMRIC) and sodium
MRI for differentiating degenerated cartilage from normal tissue. Both techniques work on the principle
that after cartilage degradation, proteoglycan loss occurs, which reduces the tissue’s negative FCD.
Consequently concentration of positively charged sodium ion (Na⁺) declines inside the tissue, and this
attenuated Na⁺ signal is captured by sodium MRI. Similarly, the negatively charged Gd (DTPA)³⁻
molecules in dGEMRIC technique accumulate in high concentration in areas lacking in GAG and in low
concentrations in GAG-rich regions [30]. In the following section, this electrically driven solute
equilibration in the negatively charged articular cartilage is described using the principle of Donnan
equilibrium partitioning.

1.4.1. Donnan partition coefficient: The partition coefficient is defined as the equilibrium
concentration of unbound or free solutes inside cartilage normalized by the solute concentration in the
equilibration bath. The partition coefficient of a solute depends on its size, charge as well as on the
composition of cartilage-matrix. A neutral solute which is not sterically hindered by cartilage ECM can
have a partition coefficient of 1, which means equal solute concentrations in the tissue and the
surrounding bath at equilibrium. The negative charge of GAGs in cartilage interacts with charged solutes
and can cause exclusion of negatively charged solutes and enhanced uptake of the positively charged
ones. This charge interaction between freely moving charged solutes and charged tissue is explained by
Donnan theory, which is based on assumptions that (1) all freely moving charged species will partition
into a charged tissue according to the Boltzmann statistics and, (2) the net charge in the tissue is zero by
electroneutrality (i.e. the sum of tissue fixed charge density and the mobile carrier concentrations). This
means that at physiological conditions, Na\(^+\) ions partition up inside the cartilage while Cl\(^-\) ions will partition down.

Transport of large sized solutes into tissue is sterically hindered and therefore will result in partition coefficients smaller than 1. Thus the size and charge of the solute as well as tissue fixed charge density are important parameters affecting partition coefficients. For example, Maroudas et al. [31,32] showed that serum albumin (MW 69kDa, diameter=7nm, pl 4.7) is sterically hindered in normal human cartilage, with a partition coefficient less than 0.05, which increased in OA cartilage owing to tissue fibrillation and loss of negatively charged groups.

1.4.2. Equilibrium binding: Binding of solutes to intra-tissue sites (for example, proteins binding to their cell surface receptors, or drugs/drug carriers functionalized to bind with ECM) can significantly slow down diffusion kinetics inside the tissue. It is, however, important for drugs/drug carriers to penetrate full thickness of tissue to reach their target sites and yet have a long residence time. The weak and reversible binding that can accompany electrostatic interactions provides a distinctive advantage by allowing drug carriers to penetrate through the full thickness of tissues. It is important to note that, while all charged solutes will partition within cartilage according to Donnan Equilibrium, electrostatic interactions do not necessarily cause “binding” of cationic solutes to negatively charged ECM macromolecules. The potential for binding must be determined experimentally on a case by case basis for each cationic solute (e.g., drug carrier) of interest. This is further discussed in Chapters 2 and 3.

1.5. Thesis outline

With the goal of developing particle-based drug delivery mechanisms for local treatment of OA, the objectives of Chapter 2 are (i) to determine the size range of solutes that can penetrate and diffuse through normal and osteoarthritic cartilage, and (ii) to investigate the effects of electrostatic interactions on solute uptake, partitioning and binding within cartilage by using a positively charged protein, Avidin. Avidin, due to its optimal size and positive charge exhibited ideal characteristics of a nanoparticle for drug delivery into negatively charged cartilage.

Based on our understanding of size and charge dependent transport of particles inside cartilage, we propose three particle based drug delivery mechanisms in Chapter 3. The effects of increasing net positive charge on cationic particles on their diffusion kinetics and retention inside cartilage are also discussed. Furthermore, governing equations to model transport kinetics inside the joint are presented.
Next, our goal was to use Avidin as a drug delivery vehicle and thus we quantified its transport kinetics, uptake and retention (half-lives) in different knee joint tissues of healthy small (rats) and large (rabbits) animals in vivo. Chapters 4 presents data from small rat models and also highlights the importance of using larger animals with thicker cartilage for understanding transport mechanisms as diffusion and diffusion-reaction kinetics is a function of the square of the tissue thickness. Chapter 5 discusses Avidin kinetics in rabbits, a larger animal model. We also evaluate the dose dependent response of Avidin on chondrocyte viability, GAG content and matrix biosynthesis to determine a safe useable dosage for delivering drugs.

We used dexamethasone (DEX) as an example drug for OA treatment, and in Chapter 6, we present synthesis and characterization of Avidin conjugated DEX structures using chemical linkers to enable both fast and slow DEX release. Fast release of drugs will provide patients with immediate pain and inflammation relief while the slow and sustained drug release is critical to suppress OA catabolic effects and restore normal biosynthesis levels. Their bioactivity is also tested in a cytokine (IL-1) challenged bovine cartilage explant model involving 3 weeks of in vitro organ culture.

Chapter 7 presents the experimental design for preclinical evaluation of safety and efficacy of single dose Avidin-DEX compound using anterior cruciate ligament transection (ACLT) injury model of post traumatic osteoarthritis in mature rabbits. This study uses 60 rabbits for evaluating efficacy at two time points: 3 weeks and 9 weeks post-surgery. This work is currently ongoing but we will present some data from the 3 weeks study. Chapter 8 will outline major conclusions from this thesis, highlight outstanding questions and also identify potential future directions.

1.6. References


Table 1.1. Potential drugs for intra-articular therapy under experimental/clinical trial phase

<table>
<thead>
<tr>
<th>Drug type</th>
<th>Examples</th>
<th>Molecular Weight</th>
<th>Mechanism of therapy</th>
<th>Target location inside joint</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pain relief</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. NSAIDs</td>
<td>- Ibuprofen (Advil, Morten)</td>
<td>&lt;500Da</td>
<td>Inhibit the COX enzymes, which are located in blood vessels of joint capsule and subchondral bone</td>
<td>Vasculature of the joint capsule (synovial lining) and cartilage-bone interface</td>
</tr>
<tr>
<td>2. Monoclonal antibodies against Nerve growth factors (Anti-NGF)</td>
<td>- Tanezumab (Pfizer) - Fluranumab (J&amp;J)</td>
<td>150Kda</td>
<td>NGF is produced by OA synovial cells &amp; chondrocytes, and acts directly on sensory neurons. Anti NGF binds to and inhibits NGF</td>
<td>Free nerve endings in soft tissues like patella ligament and below the synovial layer</td>
</tr>
<tr>
<td>Biologies</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Monoclonal antibodies</td>
<td>TNFα inhibitors*</td>
<td>150Kda</td>
<td>Prevents TNF from binding with its cell surface receptors by competitively binding with TNF</td>
<td>Close to the chondrocyte surface. Majority chondrocytes found in the deep zones of cartilage</td>
</tr>
<tr>
<td>2. Receptor antagonists</td>
<td>IL1Ra Receptor Antagonist*</td>
<td></td>
<td>Competitively binds with IL-1 or TNFα cell surface receptors thereby preventing these cytokines to bind</td>
<td></td>
</tr>
<tr>
<td>Corticosteroids</td>
<td>Salts of dexamethasone, triamcinolone, prednisone</td>
<td>&lt;1000Da</td>
<td>Intracellular GC receptors</td>
<td>Deep zones of cartilage</td>
</tr>
<tr>
<td>(Anti-catabolic effects in low doses; currently used in high doses for pain-inflammation relief only for OA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth factors</td>
<td>IGF-1, FGFs, BMPs</td>
<td>10-20kDa</td>
<td>Bind with the cell surface growth factor receptors</td>
<td>Deep zones of cartilage</td>
</tr>
<tr>
<td>(Tissue regeneration, experimental phase)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protease or Pro-protein convertase inhibitors</td>
<td>Inhibitors of MMP13, ADAMTS-5, Cathepsins, PACE 4</td>
<td>&lt;1000Da</td>
<td>Bind with the catalytic zinc atom at the MMP active site (for MMP inhibitors)</td>
<td>Both joint capsule space &amp; deep zones of cartilage</td>
</tr>
<tr>
<td>Hyaluronon or lubricin based products for joint lubrication</td>
<td>Gel-one (Zimmer), Synvisc-one (Sanoﬁ) Approved for OA</td>
<td>2000-6000kDa</td>
<td>Lubricin (PRG4) is synthesized by chondrocytes in SZ of cartilage. Type B cells of synovium make SF</td>
<td>Synovial fluid, joint capsule, synovial membrane, superficial zone of cartilage</td>
</tr>
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*Currently used for RA treatment systemically
### Figures

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<td><img src="image1.png" alt="Diagram 1" /></td>
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<td>TNFα monoclonal antibodies (eg., Humira) competitively binds with TNFα, thereby preventing TNFα from binding to cell surface receptors.</td>
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<td>Insulin like Growth Factor (IGF-1) binds to the chondrocyte IGF-1 receptor and transduces signals via the IRS-1/Pi3K/Akt pathway to regulate protein synthesis.</td>
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<td>Glucocorticoid receptors (GR) are intracellular. Eg., dexamethasone diffuses inside the cell and binds to GCs to block cytokine induced catabolic activity.</td>
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**Figure 1.1** Examples of mechanisms of interaction between some of the potential OA therapeutics and their intracellular or extracellular target binding sites.
Figure 1.2. Depth dependent properties of articular cartilage: densities of chondrocytes and negatively charged aggrecan aggregates increase with depth into the tissue from the superficial zone (SZ). SZ forms 10-20% of the total tissue thickness, MZ (middle zone) is 40-60% and the DZ (deep zone) is 30-40% of total tissue thickness. Aggrecan aggregate is a large 300MDa macromolecule comprising of HA as the core protein to which 3MDa aggrecan monomers are attached non-covalently at the G1 domain. Each aggrecan monomer has negatively charged chondroitin and keratan sulfate GAG chains that are separated from one another along the core protein by 2-3 nm, causing large electrostatic repulsion forces since the electrical Debye length is ~1 nm at physiological ionic strength. Also shown is the anatomy of the knee joint and different soft tissues.
Chapter 2 Avidin as a model for charge driven transport into cartilage and drug delivery for treating early stage post-traumatic osteoarthritis*

Local drug delivery into cartilage remains a challenge due to its dense extracellular matrix of negatively charged proteoglycans enmeshed within a collagen fibril network. The high negative fixed charge density of cartilage offers the unique opportunity to utilize electrostatic interactions to augment transport, binding and retention of drug carriers. With the goal of developing particle-based drug delivery mechanisms for treating post-traumatic osteoarthritis, our objectives were, first, to determine the size range of a variety of solutes that could penetrate and diffuse through normal cartilage and enzymatically treated cartilage to mimic early stages of OA, and second, to investigate the effects of electrostatic interactions on particle partitioning, uptake and binding within cartilage using the highly positively charged protein, Avidin, as a model. Results showed that solutes having a hydrodynamic diameter $\leq 10$ nm can penetrate into the full thickness of cartilage explants while larger sized solutes were trapped in the tissue’s superficial zone. Avidin had a 400-fold higher uptake than its neutral same-sized counterpart, NeutrAvidin, and $>90\%$ of the absorbed Avidin remained within cartilage explants for at least 15 days. We report reversible, weak binding ($K_D \approx 150 \mu$M) of Avidin to intra-tissue sites in cartilage. The large effective binding site density ($N_T \approx 2920 \mu$M) within cartilage matrix facilitates Avidin’s retention, making its structure suitable for particle based drug delivery into cartilage.

* This chapter is an edited version of the following publication:

2.1. Introduction

As discussed in Chapter 1, it is essential for a drug delivery system to enable rapid drug penetration throughout cartilage and to facilitate retention and sustained delivery to specific cell and matrix targets within the tissue. Recent research has focused on drug-encapsulating polymeric particles for use by intra-articular injection [1, 2]. Their effectiveness depends on their ability to enter the dense extracellular matrix (ECM) of cartilage and to be retained over time. A variety of particles have been explored in vitro and in vivo [3–7] but the effects of particle size and surface morphology on their penetration, binding and retention within cartilage are less well understood [8]. The relative utility of 15 nm micelles vs. 138 nm liposomes was recently reported, showing the need to further differentiate between size and structure [9].

The present study focuses on developing particle based drug delivery mechanisms for treating PTOA by investigating the effects of particle size and surface charge on transport, binding and retention within cartilage. Cartilage is an avascular tissue having a dense ECM of collagen fibrils, aggrecan proteoglycans containing highly negatively charged glycosaminoglycan (GAG) chains, and many other extracellular proteins which are continuously synthesized by the low density of chondrocytes. The type II collagen network mesh size is 60-200 nm [10], while the distance between GAG chains on aggrecan is only 1-2 nm apart from each other [11]. Aggrecan density increases with depth into cartilage from the surface (superficial) zone and restricts the ability of solutes to penetrate and diffuse within cartilage [12]. Maroudas et al. [12, 13] showed that serum albumin (MW 69 kDa, diameter~7 nm, pI~ 4.7) was sterically hindered in normal human cartilage, with a partition coefficient less than 0.05, which increased in OA cartilage. Immunoglobulin (IgG) antibodies (MW~160 kDa) were sterically excluded by cartilage ECM. However, Fab antibody fragments (e.g., an anti-IL-6 Fab, 48 kDa [14]) can diffuse into bovine and human cartilage over 3 days. Fab uptake was higher in the superficial compared to deeper zones, suggesting that transport is dependent on the aggrecan content. These reports suggest the need for investigating nano-sized particles for delivery into cartilage.

While transport of solutes is size and shape dependent, binding within cartilage ECM is also modulated by particle surface properties including charge. The high negative fixed charge density of cartilage is known to regulate Donnan partitioning and binding of proteins, growth factors and other macromolecules. For example, negatively charged albumin partitions downward [12], while the positively charged growth factor IGF-1 (pI 8.5) partitions upward into cartilage [15]. Here, we investigate Avidin, a globular and highly glycosylated protein, as an example of a structure that due to its size (MW 66 kDa,
diameter ~7 nm) and high positive charge (pI 10.5) may offer unique advantages for rapid uptake and binding within the tissue. Avidin has been previously investigated for targeted delivery into tumors [16]; results showed enhanced tissue and cellular uptake and binding due, in part, to electrostatic interactions.

With the goal of developing particle-based drug delivery mechanisms for treating PTOA, the objectives of this study were (1) to determine the size range of a variety of solutes that could penetrate and diffuse through normal and enzymatically treated cartilage to mimic early stages of OA, and (2) to investigate the effects of electrostatic interactions on solute uptake, partitioning and binding within cartilage by using the highly positively charged protein, Avidin.

2.2. Materials and Methods

In a series of transport studies, cartilage disks were incubated in medium containing a range of fluorescently tagged solutes of varying size and charge. Cross-sections of the cartilage were then imaged using confocal microscopy to determine the depth of penetration and the spatial distribution of each solute type within the tissue. In separate experiments to obtain a measure of total solute uptake, cartilage disks were equilibrated in solutions of selected solutes and then desorbed into phosphate buffered saline (PBS) baths. The measured fluorescence in the absorption and desorption baths were used to quantify the equilibrium uptake ratio, partition coefficient, and equilibrium binding properties of these solutes within the tissue. Additional studies of non-equilibrium transport through cartilage disks enabled estimation of the effective diffusivity of selected solutes within cartilage.

2.2.1. Bovine cartilage harvest and culture

Cartilage disks were harvested from the femoropatellar grooves of 1-2 week old bovine calf knee joints (obtained from Research 87, Hopkinton, MA) as described previously [17]. Briefly, cylindrical cartilage disks (3 mm or 6 mm diameter) were cored using a dermal punch and then sliced to obtain the top 1mm of cartilage with intact superficial zone. Cartilage disks for all treatment groups were matched for depth and location along the joint surface. The disks were then pre-equilibrated in PBS (without Ca²⁺/Mg²⁺) supplemented with protease inhibitors (Complete Protease Cocktail tablet in 50 mL PBS, Roche Applied Science, IN) in a 37°C, 5% CO₂ incubator for 24-48h.

2.2.2. Solutes types

a) Size exclusion studies: We used solutes having a wide range of sizes from ~0.9 nm to 15 nm diameter: (i) fluorescein isothiocyanate (FITC, 389.3 Da, diam ~0.9 nm), (ii) FITC-dextran (8 kDa,
hydrodynamic diameter 4.3 nm), (iii) FITC-dextran (40 kDa, diameter ~10 nm (all from Sigma Aldrich, MO); (iv) FITC-conjugated NeutrAvidin, an electrically neutral globular protein at pH 7 (60 kDa, diameter ~7 nm; Invitrogen, CA) and (v) Cd-Se Quantum Dots 15 nm in diameter (Red, synthesized at MIT [18]).

**b) Binding/Retention studies:** Effects of electrostatic interactions on solute transport, uptake and binding were investigated by using (i) FITC-conjugated and non-labeled Avidin (pI 10.5, 66 kDa, diameter ~7 nm, Invitrogen, CA), the positively charged counterpart of NeutrAvidin, and (ii) amine functionalized 15 nm diameter Cd-Se quantum dots (QDs) (Qdot®565, Green, Invitrogen, CA, USA). FITC-dextran (8kDa) was dialyzed using 1 kDa MW cut off dialysis tube (Float-A-Lyzer G2, SpectrumLabs Inc., CA) and all other solutes were dialyzed using 3 kDa cutoff MW centrifugal filter (Amicon Ultra-4, Millipore Corp, MA) to determine the amount of free FITC; the fluorescence readings of these solutions after dialysis indicated negligible amounts of free FITC. The solute types with their physical properties are listed in Table 2.1.

### 2.2.3. Transport configuration for confocal microscopy imaging

A special poly(methyl methacrylate) (PMMA) transport chamber was designed to study one-way diffusion of solutes entering into cartilage from the tissue’s superficial zone (SZ) (i.e., transport in the X direction in Fig. 2.1). The chamber walls were treated with casein to block non-specific binding of solutes to PMMA surfaces. Pre-equilibrated cartilage disks (6 mm diameter, 1 mm thick) were first cut in half, and the half-disk specimens were placed within holding slots machined into the chamber (Fig 2.1A). The upstream chamber side facing the superficial zone was filled with 45 μl of a known concentration of solute in 1X-PBS solution supplemented with protease inhibitors (Roche Applied Science, IN); the downstream chamber side was filled with 45 μl of 1X-PBS containing protease inhibitors alone. The chamber was then placed in a petri dish containing DI water, covered (to minimize evaporation), and placed on a slow-speed rocker inside an incubator at 37°C to minimize stagnant layers at cartilage surfaces.

After 24-96 h, the cartilage half-disks were removed from the bath, gently rinsed in 1X PBS, and surface fluid along with any non-absorbed solutes were gently removed with Kimwipes. Using a scalpel, a slice (100-200 μm thick) was then cut from the center of each disk (Fig. 2.1B). The middle region of the slice (shown by the dotted boundary) was imaged in the X-Y plane using a confocal microscope (Nikon TE2000-U) at 10X magnification to identify the penetration and X-directed solute concentration profile within the tissue. Appropriate optical filters were chosen to eliminate auto-fluorescence of cartilage at the
settings used for imaging. For desorption studies, the solute solution was removed from the chamber of Fig. 2.1 and replaced with 1X or 10X PBS containing protease inhibitors. To ensure proper image comparison, solute concentrations were chosen such that the FITC concentration in each solution was identical, thereby giving equal fluorescence intensities. Nominal concentrations for the absorption baths were 2.5 μM (FITC), 125 μM (FITC-dextran, 8 kDa), 25 μM (FITC-dextran, 40kDa), 18 μM (Avidin), and 30 μM (NeutrAvidin). 100 μM (FITC-Dextran, 40 kDa) was also used for a separate 24-96 h transport study (Fig. 2.2D-F). The concentrations for the two types of QD solutions were chosen such that they exhibited equal fluorescence intensity.

2.2.4. Quantitative analysis of solute uptake into cartilage

a) Quantum Dot Uptake using Induced Coupled Plasma Measurement: The total uptake of QDs into cartilage half disks was measured via quantification of the amount of cadmium (111Cd) present in the tissue and the absorption/desorption baths that were collected immediately after each QD uptake experiment. (Cd is present in the core of QDs). Inductively coupled optical-emission spectrometry (ICP-OES) was performed using a Horiba Jobin Yvon Activa ICP OES (Horiba Scientific, NJ) to quantify the amounts of 111Cd using a previously published method [19]. The sum of final amounts of Cd in the bath and the cartilage half disks corresponded to the initial amount of Cd in the starting 45 μl of QD-PBS upstream solution. The Cd amounts were converted into QD concentrations using calibration plots made for each QD studied. The background amount of Cd in fresh, untreated cartilage was measured to be zero.

b) Equilibrium uptake of Avidin and NeutrAvidin: 3 mm diameter, 1 mm thick cartilage explants were incubated for specific times in 300 μl of known concentration (3μM) of FITC-Avidin and FITC-NeutrAvidin, supplemented with protease inhibitors at 37°C in a 96 well plate format. After removal from the absorption baths, the disks were rinsed, gently wiped and then incubated in 1X or 10X PBS supplemented with protease inhibitors for 24 h or longer as specified. At the end of the experiment, the surfaces of each disk were quickly blotted with Kimwipes and the wet weight was measured. The disks were then lyophilized and the dry weight was measured; the water weight was calculated from the tissue wet and dry weights. The fluorescence signal in the absorption and desorption baths was quantified using a plate reader (1400 Wallace Victor, PerkinElmer, MA); the solute content inside the cartilage disk was determined from the difference between the fluorescence reading of the absorption/desorption baths before and after incubation. In establishing standard curves, the fluorescence intensities and solute concentrations for both FITC-Avidin and FITC-NeutrAvidin were found to be linear with bath concentration. The solute uptake ratio was calculated as the concentration of the FITC-solute in the
cartilage (per intra-tissue water weight) normalized to the concentration of FITC-solute in the equilibration bath.

2.2.5. Effect of sGAG depletion on solute uptake

To understand the effects of the negatively charged glycosaminoglycan (GAG) chains within cartilage matrix on solute uptake and binding, groups of cartilage disks (3 mm diameter, 1 mm thick) were treated with either chondroitinase-ABC (Sigma Aldrich, MO, USA), or trypsin (Invitrogen, CA). Chondroitinase-ABC digests and removes GAG chains (predominantly the chondroitin sulfate GAG chains of the highly abundant aggrecan proteoglycans in cartilage) while the protease, trypsin, cleaves the core proteins of aggrecan and other GAG-containing proteoglycans and glycoproteins. However, both treatments leave cartilage’s collagen network intact [20]. The dimethyl-methylene blue (DMMB) dye binding assay [21] was used to quantify the content of sulfated GAG (sGAG) remaining in the disks after enzyme treatment as well as that lost to the medium as previously described [22], and the percentage of GAG removed by specific enzyme treatments was thereby calculated. For one series of experiments, a 24 h chondroitinase-ABC treatment (0.1U/ml in 0.15 M NaCl, 0.05 M Na phosphate, pH 7.2 for 24 h at 37°C) was used, resulting in 38.6% (~40%) depletion of sGAG, primarily from the outer tissue surfaces, which mimics the initial GAG loss caused by traumatic joint injury in vivo [23] and in models of cartilage injury in vitro [24]. A second group of disks was treated with trypsin (1 mg/ml, in 0.15 M NaCl, 0.05 M Na phosphate, pH 7.2 for 24 h at 37°C). Previous studies showed that treatment of similar bovine calf cartilage disks with 1 mg/ml trypsin caused nearly complete loss of measurable sGAG by 24 h [25]. After enzyme treatments, the disks were washed three times in fresh PBS. Uptake experiments were then conducted using solute-PBS solutions containing protease inhibitors to minimize any additional protease activity. The transport and binding properties were then compared with that in the normal cartilage.

2.2.6. Transport measurements for effective diffusivity

Real-time measurement of diffusive transport of Avidin and NeutrAvidin through young bovine cartilage disks (with intact superficial zone) was measured using a diffusion chamber consisting of two compartments as described previously [15]. Groups of three cartilage disks (6 mm diameter, 400 μm thick) were clamped by O-rings between the two compartments of the diffusion chamber (with total exposed tissue area for transport of 0.28 cm²/disk), such that solute transport from the upstream compartment into and across the cartilage disks, simultaneously, could occur only from the superficial zone of the cartilage (schematic shown in the inset of Fig. 2.7). The compartments were treated with casein to prevent non-specific adsorption of solutes to the chamber surfaces. Each compartment was then
filled with 25 mL of 0.15 M NaCl with protease inhibitors and maintained at 20 °C. At starting time $t = 0$, FITC tagged Avidin or NeutrAvidin was added to the ‘upstream’ compartment, resulting in transport through the tissue into the downstream compartment. The baths in both compartments were magnetically stirred to minimize the effects of stagnant layers at the solution-tissue interfaces. Aliquots were taken from each chamber at different time intervals, and fluorescence was measured using a plate reader.

We note that the compartment volume of 25 mL is approximately 1,000 X larger than the volume of the cartilage plugs in the configuration of Fig. 2.7 (inset). As a result, the boundary solute concentration at the cartilage-solution interfaces, both upstream and downstream, remained to within ~5% of their starting ($t=0$) values throughout the course of these transport experiments. This configuration thereby focuses on quantitation of steady state solute flux and assessment of effective solute diffusivity within cartilage. In contrast, the transport chamber compartment volumes in the configuration of Fig. 2.1 are relatively smaller and closer to the volume of the cartilage disks, a configuration that more closely approximates the relative solid-fluid volumes of cartilage and adjacent synovial fluid in joints in vivo. (For example, human tibial plateau cartilage volume is ~4 mL [26], and knee joint synovial fluid volume ranges from ~1-4 mL [27]).

2.2.7. Statistical analysis:

Data on solute uptake and desorption (e.g., Figs. 2.31-J and 2.5A-C) are presented as Mean ± SEM. We used the general linear mixed effects model with animal as a random variable for analysis followed with the Tukey’s test for comparisons between multiple treatment conditions. Fig. 2.3 data are derived from 2 different animals. A total of $n=3$ cartilage samples per animal in each treatment condition were used. The mean represents the average of 6 samples per treatment condition, as there was no effect of animal. Figure 2.5 data are derived from 3 different animals for the normal cartilage condition and 1 animal for the 40% GAG depleted condition. A total of $n=6$ cartilage samples per animal in each treatment condition was used; the mean represents the average of 18 samples per treatment for the normal cartilage condition (since there was no effect of animal) and the average of 6 samples per treatment for the 40% GAG depleted condition. We used $p<0.05$ for statistical significance.

2.3. Results

2.3.1. Effect of solute size and molecular structure on transport into cartilage:

Transport studies with FITC (389 Da, diameter ~0.9 nm) and FITC-dextran (8 kDa, diameter ~4.3 nm) showed that particles with hydrodynamic diameter < 5 nm penetrated throughout the full thickness (1
mm) of the cartilage explant within 24 h, while a penetration gradient was still evident at 24 h for 40 kDa FITC-dextran (diameter ~10 nm) (Fig. 2.2A-C). The relative fluorescence intensity vs. penetration distance into cartilage is plotted below the images, illustrating size-dependent transport. 40 kDa FITC-dextran solutes (expandable polysaccharide coil) diffused into the major part of the cartilage thickness in 4 days (Fig. 2.2D-F), while the globular protein, NeutrAvidin (MW 60 kDa, diameter ~7 nm) penetrated approximately half the sample thickness (Fig. 2.2G-I). 15 nm diameter QDs were trapped in the cartilage superficial zone and penetrated only the first 40-50 μm of tissue in 24 h (Fig. 2.3A, 2.3C). The penetration depth of these QDs did not change even by 6 days (data not shown), suggesting that 15 nm diameter particles are too large to penetrate through the complex meshwork of cartilage matrix. However, trypsin treated samples allowed the 15 nm QDs to penetrate through the full thickness of the cartilage disk in 24 h (Fig 2.3E, 2.3G). DLS measurements (Malvern Zetasizer-ZS90 equipped with a He-Ne laser) showed no aggregation of QDs at 37°C over this time course.

2.3.2. Effect of particle surface properties on uptake, retention and binding within cartilage:

Amine functionalized QDs (GREEN) did not desorb after 24 h in 1X PBS while the QDs with no functional group (RED) did (Fig. 2.3B, 2.3D). Desorption in 10X PBS significantly reduced the retention of green QDs in the cartilage disks from 64% to 0.4% of the absorbed amount in 24 h (Fig. 2.3J). Trypsin treated samples exhibited significantly lower retention (~40%) compared to normal cartilage (~64%) but retained similar amounts with 1X and 10X desorption (Fig 2.3J), suggesting charge based interactions. Matrix degradation due to trypsin treatment significantly enhanced the penetration and uptake for both types of QDs, as expected (Fig 2.3E, G, I).

To further explore the effects of electrostatic interactions, the transport and binding properties of Avidin (a highly positively charged globular protein) were compared to that of its neutral counterpart, NeutrAvidin. Despite their similar sizes, NeutrAvidin penetrated only half the specimen thickness in 4 days (mean uptake ~0.44) while Avidin diffused through the full thickness of the cartilage (mean uptake ~183) within 1 day, resulting in greater than 400 times higher uptake of Avidin compared to NeutrAvidin (Fig. 2.4A-C; 2.5A-B). About 50% of the absorbed NeutrAvidin diffused out of the cartilage within 1 day in 1X PBS, while 96% of the absorbed Avidin remained inside the cartilage even by 15 days (the duration of the experiment conducted) in 1X PBS (Fig. 2.5C). However, a significantly higher percent (~ 69%) of the absorbed Avidin diffused out of the cartilage in 10X PBS within 24 h, suggesting effects of strong electrostatic interactions (Fig. 2.4D-E). We hypothesized that the Avidin could be binding (reversibly) to the negatively charged GAG chains in the cartilage matrix. Depletion of 40% of the cartilage sGAG (using chondroitinase-ABC) resulted in significantly reduced uptake of Avidin over a 24
h period, from a mean value of 183 in normal cartilage to 24 in GAG-depleted tissue (Fig. 2.5A). This further confirmed the effects of charge interactions and showed that the negatively charged sGAG chains of cartilage matrix play a critical role in enhancing the transport, uptake and binding properties of large, positively charged globular proteins like Avidin. The uptake of NeutrAvidin, however, increased from 0.28 in the normal cartilage to 0.54 in GAG depleted cartilage by 24 h (Fig. 2.5B), which is most likely due to the increase in the matrix pore size resulting from 40% sGAG depletion.

2.3.3. Avidin uptake as a function of bath Avidin concentration:

Based on the results of Fig 2.5, we hypothesized that Avidin could bind to sites within the cartilage. To test this hypothesis, we performed competitive binding experiments in which 3 mm diameter cartilage disks were equilibrated for 3 days in 300 μl buffer containing a fixed amount of (fluorescently labeled) FITC-Avidin (1 μM) and graded amounts of unlabeled Avidin (0, 10, 76, 100, and 203 μM). The disks were split into half disks to reduce the time needed for equilibration, which was performed in 96 well plates at 37°C. The uptake ratio, R_u, was measured and plotted versus the total bath concentration of Avidin (labeled + unlabeled, Fig. 2.6), where R_u is defined as the total concentration of Avidin inside the cartilage (bound (C_B) plus free (C_F)) per intra-tissue water weight, normalized to the Avidin concentration in the equilibration bath (C_Bath):

\[ R_u = \frac{C_B + C_F}{C_{Bath}} \]

Labeled and unlabeled Avidin were assumed to partition into the cartilage identically. At very low concentration of the labeled Avidin (≤ 1 μM), a high uptake of ~120 was observed. When unlabeled Avidin was added to the bath, both species (labeled and unlabeled) could compete for the same (constant) number of binding sites available in the tissue (site density N_T). As the concentration of unlabeled Avidin was increased, the uptake of labeled Avidin was observed to decrease dramatically (Fig. 2.6). To model the data of Fig. 2.6, we assumed a simple first-order, bimolecular, reversible reaction to describe binding of Avidin to a single dominant binding species within cartilage. We adopted a similar previously described theoretical model [15] used for characterizing the binding of soluble insulin like growth factor-1 (IGF-1) to IGF-binding proteins (IGF-BPs) that are uniformly enmeshed and fixed within cartilage matrix. According to this model, the equilibrium molar concentration of free solute (C_F), bound solute
\( C_B \), intra-tissue binding site density \( (N_T) \) and the equilibrium dissociation constant \( (K_{EQ}) \) are related by the binding isotherm:

\[
\frac{C_B}{C_F} = \frac{N_T}{K_{EQ} + C_F}
\]

(2)

which is similar in form to a Langmuir adsorption isotherm. In addition, the equilibrium partition coefficient \( (K) \) of solute, Avidin, is defined as the concentration of the free solute inside the cartilage disk (per intra-tissue water weight) normalized to the concentration of solute in the bath:

\[
K = \left( \frac{C_F}{C_{Bath-final}} \right)_{\text{unlabeled Avidin}} = \left( \frac{C_F}{C_{Bath-final}} \right)_{\text{FITC-Avidin}}
\]

(3)

In these experiments, the final Avidin bath concentration in Equation (3), \( C_{Bath-final} \), is generally different from the initial bath concentration, \( C_{Bath-initial} \), because of the very high uptake of Avidin into cartilage. The ratio of final to initial Avidin bath concentration is defined as \( f \):

\[
\left( \frac{C_{Bath-final}}{C_{Bath-initial}} \right)_{\text{FITC-Avidin}} = \left( \frac{C_{Bath-final}}{C_{Bath-initial}} \right)_{\text{unlabeled}} = f
\]

Then the partitioning of labeled and unlabeled Avidin into cartilage then becomes:

\[
C_F = KfC_{Bath-initial}
\]

(4)

Combining Equations 1-4 yields:

\[
R_U = K \left( 1 + \frac{N_T}{K_{EQ} + KfC_{Bath-initial}} \right)
\]

(5)

The theoretical curve for \( R_U \) was fit to the data of Fig. 2.6 (solid line) using nonlinear least squares; the best fit values for the three unknown parameters were, \( K = 5.9 \), \( K_{EQ} = 150.3 \mu\text{M} \) and \( N_T = 2920 \mu\text{M} \). We note that the partition coefficient \( K \) is the value of \( R_U \) in Eq. (5) in the limit of very high Avidin bath concentration (i.e., the limit in which all the binding sites are occupied by the unlabeled Avidin). Thus, \( K \) is determined by both steric hindrance and electrostatic (Donnan) interactions. (The data of Fig 2.6, re-plotted in the form of the binding isotherm of Eq. (2), is presented in Supplemental Section S1 for comparison, Fig 2S.1)
2.3.4. Characterization of non-equilibrium transport of Avidin across bovine cartilage:

The transport cell arrangement shown in the inset of Fig. 2.7 was used to measure transient transport of Avidin and NeutrAvidin into and across cartilage disks. Fig. 2.7 shows real-time measurements of the downstream concentration of FITC-Avidin (normalized to upstream concentration) that had diffused through a group of three cartilage explants in parallel. Extrapolation of the linear slope of concentration versus time (between \( t = 50 \) to \( t = 150 \) minutes) to the time axis gives the time lag \( \tau_{\text{lag}} \) to achieve the steady state flux [28] which, for Avidin, was \( \tau_{\text{lag}} \sim 35 \) min. This \( \tau_{\text{lag}} \) is related to the effective diffusivity, \( D_{\text{EFF}} \), of Avidin that characterizes the initial transport transient [28]:

\[
\tau_{\text{lag}} = \frac{\delta^2}{6D_{\text{EFF}}}
\]

where \( \delta \) is the thickness of the cartilage disk (\(-400 \mu m\)). \( D_{\text{EFF}} \) for Avidin was calculated to be \( 3.8 \times 10^{-7} \, \text{cm}^2/\text{s} \). We hypothesized that this time lag and, hence, \( D_{\text{EFF}} \) could be associated in part with the effects of binding of Avidin within the cartilage. Once this binding has reached steady state, a corresponding steady state flux would be achieved, as seen in Fig 2.7 from \( t = 35 \) min to \( t = 186 \) min. This steady state flux is expressed in terms of the steady state diffusivity \( D_{\text{SS}} \) by:

\[
\Gamma = \Phi K D_{\text{SS}} \frac{C_{\text{U}} - C_{\text{D}}}{\delta} = \Phi K D_{\text{SS}} \frac{C_{\text{U}}}{\delta}
\]

where \( \Phi \) is the tissue porosity (measured from wet and dry weights to be \( \Phi = 0.81 \)), \( K \) is the partition coefficient, and \( C_{\text{U}} \) and \( C_{\text{D}} \) are upstream and downstream bath concentrations, respectively. The time derivative of the normalized downstream concentration (slope) is related to the steady state flux by:

\[
\frac{\partial}{\partial t} \left( \frac{C_{\text{D}}}{C_{\text{U}}} \right) = \frac{\Gamma A}{V_{\text{D}} C_{\text{U}}} \approx \frac{\Phi K D_{\text{SS}} A}{\delta V_{\text{D}}}
\]

where \( A \) is the total exposed tissue area (\( 0.84 \, \text{cm}^2 \)) and \( V_{\text{D}} \) (\( 25 \, \text{cm}^3 \)) is the volume of the downstream bath. Using Eq. 8, the product \( K D_{\text{SS}} \) for Avidin was calculated to be \( 1.4 \times 10^{-5} \, \text{cm}^2/\text{s} \). Similarly, \( K D_{\text{SS}} \) for NeutrAvidin was calculated to be \( 2.3 \times 10^{-6} \, \text{cm}^2/\text{s} \), giving a ratio of \( (K D_{\text{SS}})_{\text{Avidin}} \) to \( (K D_{\text{SS}})_{\text{NeutrAvidin}} \) of \(-10 \). Avidin and NeutrAvidin are of similar size and, hence, are expected to have similar steady state diffusivity, \( D_{\text{SS}} \). The partition coefficient for NeutrAvidin, \( K_{\text{NeutrAvidin}} \) was estimated from the data of Fig. 2.5B to be \( 0.44 \). Using these values, we calculated \( K_{\text{Avidin}} \) to be \( 4.4 \) and \( D_{\text{SS}} \) to be \( 3.2 \times 10^{-6} \, \text{cm}^2/\text{s} \).

To test whether any unbound FITC was present which could affect the measurement of the total flux and thereby the estimate of diffusivity, free FITC (MW 389.3 Da) was added at \( t = 186 \) min into the
upstream bath. Almost immediately, the diffusive flux of fluorescently labeled species across the cartilage increased dramatically (Fig. 2.7). The diffusivity of free FITC was thereby estimated to be $2.8 \times 10^{-5}$ cm$^2$/s, one order of magnitude higher than the steady state diffusivity ($D_{SS}$) calculated for Avidin, implying that there was a negligible amount of free FITC present which could affect the measurement of the flux of FITC-Avidin.

Assuming that $D_{EFF}$ includes the effects of binding, modeled using first order, reversible bimolecular reaction kinetics, $D_{EFF}$ can be derived in terms of $D_{SS}$ [15]. During the initial addition of labeled Avidin, i.e., in the limit in which $C_F < K_{EQ}$, $D_{EFF}$ is related to $D_{SS}$ by [15]:

$$D_{EFF} \approx D_{SS} \left(1 + \frac{N_F}{K_{EQ}}\right)^{-1}$$

(9)

From the best fit values (section 3.2), $1 + \frac{N_F}{K_{EQ}} = 20.4$. Using $D_{EFF} \approx 3.8 \times 10^{-7}$ cm$^2$/s (from figure 2.7), $D_{SS} = 7.7 \times 10^{-6}$ cm$^2$/s, which is on the same order of magnitude as that calculated from the transport cell experiment. (See Table 2.2 for transport properties estimated for Avidin.)

### 2.4. Discussion

Traumatic joint injuries can result in damage to many soft and hard tissues. While joint cartilage sometimes remains undamaged, varying changes are observed, from subtle microdamage to the matrix (not visualizable by MRI or arthroscopy) up to overt fibrillation and cracks [29]. Trauma simultaneously increases the levels of inflammatory cytokines in the synovial fluid, which can predispose even undamaged cartilage to rapid chondrocyte-mediated proteolysis and loss of aggrecan and other matrix molecules within the first days/weeks post-injury [23,29-32], eventually leading to PTOA. With the need to identify drug-carrying nanoparticles that can penetrate rapidly within cartilage to provide sustained delivery of drugs to cell and matrix targets throughout the tissue, we studied a range of particle sizes and types to test their ability for rapid and sustained uptake.

We found that deep penetration into normal (undamaged) cartilage required particle diameters $< 10$ nm (Figs. 2.2-2.4). If injected intra-articularly, in-vivo clearance would define a practical lowest size limit [33,34]. While 15 nm diameter particles were sterically hindered and trapped in the superficial zone of normal cartilage, they could penetrate into the deeper zones of proteoglycan-depleted cartilage (Fig. 2.3), consistent with previous reports of solute penetration [12,13,35–39]. Such larger sized particles can be used for drug delivery if they can be functionalized to specifically bind within the superficial zone of
cartilage. As these particles gradually degrade, they could release drugs which could then diffuse and/or bind within the cartilage over time. We demonstrated this approach by using amine functionalized QDs; however, the specific binding mechanism in this case is not apparent.

The high negative fixed charge density of cartilage offers the unique opportunity to utilize electrostatic interactions to augment transport, binding and retention of drug carriers. Recently, transport into cartilage explants of a small cationic peptide therapeutic was investigated (Arg-Tyr-Lys-Arg-Thr; 760 Da, net charge +3; pI = 11). The concentration of the peptide was indeed higher in cartilage due to Donnan (electrostatic) partitioning (as would be expected), but the peptide did not bind within the cartilage and therefore rapidly diffused out [39]. Reversible binding to intra-tissue sites is necessary to maintain enhanced intra-tissue concentration for sustained local delivery, and separate experiments must be performed for any given nanoparticle to test whether electrostatic interactions can simultaneously affect non-equilibrium transport and equilibrium uptake (the latter associated with binding of solutes to matrix and/or upward Donnan partitioning of unbound solutes into intra-tissue fluid).

In this study, we report that Avidin’s structure due to its size and high positive charge exemplifies distinct advantages for a particle-based drug delivery system. Avidin penetrated throughout the full thickness of cartilage explants within 24 h, while the same-sized neutral counterpart, NeutrAvidin, took four days to penetrate into half the thickness (Figs. 2.2 and 2.4). Avidin showed a 400 times higher equilibrium uptake compared to NeutrAvidin in normal cartilage. Additionally, Avidin was retained within cartilage for at least 15 days, while NeutrAvidin was mostly released when explants were placed in a 1X PBS desorption bath for 24 h (physiological ionic strength) (Fig. 2.5). When placed in high salt (10X PBS), Avidin, too, was readily desorbed due to the shielding of electrostatic interactions.

The transport of a large, positively charged molecule like Avidin through negatively charged cartilage is influenced by three phenomena (i) steric hindrance from the dense tissue ECM (characterized by $D_{\text{SS}}$, Eq. (7)), (ii) binding to the intra-tissue sites (characterized by $D_{\text{EFF}}$, which is a function of $D_{\text{SS}}$, $N_T$, and $K_{\text{EQ}}$, Eq. (9)), and (iii) Donnan partitioning of unbound Avidin due to electrostatic interactions (characterized by $K$, Eqs. (3,7)). Upon initial addition of Avidin to the bath, electrostatic interactions would result in high upward Donnan partitioning of Avidin at the solution-cartilage interface (through K). The resulting steep intra-tissue concentration gradient would greatly enhance transient transport of Avidin into the tissue compared to that of similarly sized but neutral, NeutrAvidin, as observed (Figs. 2.4 and 2.5). At final equilibrium, the high uptake of Avidin could be due to either intra-tissue binding and/or tissue-wide upward Donnan partitioning. To distinguish between these effects, we performed additional competitive binding experiments (Fig. 2.6) which suggest that Avidin binds weakly and reversibly to sites
in cartilage with a $K_D \sim 150 \mu M$. The predicted high binding site density ($N_T \sim 2920 \mu M$) is consistent with the high concentration of intra-tissue GAGs, and explains Avidin’s long retention time (~15 days) inside the tissue.

Further evidence of the presence of binding interactions is provided by the non-equilibrium transport experiments of Fig. 2.7: the measured diffusion lag time ($\tau_{\text{lag}}$) suggests that binding slowed the initial transport of Avidin into cartilage compared with the final steady state diffusive transport across the tissue. The effective diffusivity $D_{\text{eff}}$ (which includes the effects of binding from Eq. (9)) was estimated from the measured $\tau_{\text{lag}}$ (Eq. (6)); $D_{\text{eff}}$ was an order of magnitude less than $D_{\text{ss}}$, the diffusivity after binding had reached steady state. Nevertheless, this weak, reversible binding did not inhibit Avidin’s rapid penetration into the full depth of the explants because of the steep intra-tissue concentration gradient caused by Donnan partitioning.

Because of the importance of electrostatic interactions in the uptake and transport of Avidin in cartilage, we used several approaches to estimate the effective net charge of the Avidin tetramer responsible for the experimental observations presented. First, the amino acid structure of Avidin suggests a net tetrameric charge of +20 based on the net excess of basic over acidic residues (see Supplementary Section S2). However, this estimate assumes that all such residues are ionized in aqueous solution and that none of these residues are internal (buried) and thereby inaccessible to charge-charge interactions within the cartilage matrix. Furthermore, this estimate neglects the effects of Avidin glycosylation which could add additional negative charge groups to the total sum. Therefore, we also applied Donnan equilibrium theory to the experimental results of Figs. 2.5B and 2.6 to calculate Avidin charge (Supplementary Section S3). Donnan theory [40] is based on the assumptions that (1) all freely moving charged species (i.e., Avidin and bath ions) will partition into a charged tissue according to Boltzmann statistics and, (2) the net charge in the tissue is zero by electroneutrality (i.e., the sum of the tissue fixed charge density and mobile carrier concentrations). By fitting the Donnan theory to the equilibrium uptake data of Fig. 2.5A, the effective net charge of Avidin was calculated to be +6.2. This value is very close to the effective charge of +7.3 that we obtained by using the reported zeta potential of Avidin [41] in the Grahame equation for spherical particle electrokinetics (Supplementary Section S.4). A final upper estimate of effective net charge was obtained by assuming that the uptake of Avidin in the experiments of Figure 2.6 was entirely due to Donnan partitioning into cartilage in the complete absence of binding to intra-tissue sites (Supplementary Section S.5, Fig. 2S.2). This estimate gave a net charge of +13 to +14.
The loss of the negatively charged GAGs soon after a joint injury might limit the extent of electrostatic interactions available for binding and retention of cationic solutes within the tissue. We simulated such a post-injury condition using chondroitinase-ABC to remove ~40% of explant GAG chains. Avidin uptake was not as high as in normal cartilage, but still achieved a very high value of 24 (Fig. 2.5A), and remained bound to intra-tissue sites even when placed in 1X PBS desorption bath. Together, these observations suggest that Avidin may be useful in-vivo as a model drug delivery mechanism for cartilage, and that therapeutic drug carriers with properties similar to Avidin (~7nm diameter and a high positive charge) might enable rapid, high uptake inside the cartilage, bind within the tissue, and thereby providing sustained local drug delivery.

2.5. Conclusion

Based on our results, we propose a mechanism for nanoparticle based drug delivery into cartilage involving highly positively charged drug carrying particles with diameter < 10 nm, which can diffuse through the full thickness of cartilage and are capable of binding to sites within the ECM. Avidin provides an excellent example of such a nanoparticle, which could potentially release and deliver low molecular weight functionalized drugs. A second approach could utilize slightly larger sized particles that can bind within the superficial zone of cartilage and then release functionalized drugs. The QD data of Fig. 2.3 exemplifies this approach. As these particles gradually degrade, they could release drugs which could then diffuse and/or bind to sites within the cartilage over time. With both approaches, electrostatic interactions between positively charged nanoparticles and the negative fixed charge of cartilage ECM can be optimized to augment transport, uptake and intra-tissue binding of such drug carriers.

2.6. References

Tables and figures

Table 2.1. Physical properties of different solute types used for diffusion studies.

<table>
<thead>
<tr>
<th>Solute</th>
<th>Average Molecular Weight (Da)</th>
<th>Hydrodynamic diameter (nm)</th>
<th>Electric Charge in solution</th>
<th>Molecular Structure</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescein Isothiocyanate (FITC)</td>
<td>389.3</td>
<td>10-0.9</td>
<td>Negative</td>
<td>Rigid molecule</td>
<td>[54] Sigma Aldrich</td>
</tr>
<tr>
<td>8kDa FITC-Dextran</td>
<td>8000</td>
<td>4-4.3†</td>
<td>Negative</td>
<td>Polysaccharide, expandable coil</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>40kDa FITC-Dextran</td>
<td>40,000</td>
<td>9-10†</td>
<td>Negative</td>
<td>Polysaccharide, expandable coil</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Avidin-FITC</td>
<td>66,000*</td>
<td>7</td>
<td>Positive</td>
<td>Globular protein; tetrameric structure; glycosylated</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>NeutrAvidin-FITC</td>
<td>60,000*</td>
<td>7</td>
<td>Neutral</td>
<td>Globular protein; tetrameric structure; non-glycosylated</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Green Cd-Se Quantum Dots</td>
<td>X</td>
<td>15</td>
<td>Slight Positive</td>
<td>Spherical; Cd-Se core functionalized with amine- derivatized PEG</td>
<td>[18] Invitrogen</td>
</tr>
<tr>
<td>Red Cd-Se Quantum Dots</td>
<td>X</td>
<td>15</td>
<td>Neutral</td>
<td>Spherical; Cd-Se core functionalized with PEG</td>
<td>[19]</td>
</tr>
</tbody>
</table>

*MW of the tetrameric structure
†Hydrodynamic diameter in free solution when dextran is coiled [42]

Table 2.2. Transport properties estimated for Avidin from the transport cell and binding isotherm.

<table>
<thead>
<tr>
<th>Avidin Transport Properties</th>
<th>Binding Isotherm</th>
<th>Transport cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Partitioning, $K_{Avidin}$</td>
<td>~6</td>
<td>~4.4</td>
</tr>
<tr>
<td>Binding density ($N_T$)</td>
<td>~2920 µM</td>
<td>X</td>
</tr>
<tr>
<td>Dissociation Constant ($K_{eq}$)</td>
<td>~150 µM</td>
<td>X</td>
</tr>
<tr>
<td>Effective Diffusivity ($D_{eff}$)</td>
<td>X</td>
<td>3.8 x 10⁻⁷ cm²/s</td>
</tr>
<tr>
<td>Steady State Diffusivity ($D_{ss}$)</td>
<td>7.7 x 10⁻⁶ cm²/s</td>
<td>3.2 x 10⁻⁶ cm²/s</td>
</tr>
<tr>
<td>$D_{ss} = D_{eff} \left(1 + \frac{N_T}{K_{eq}}\right)$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Effective Charge</td>
<td>+6</td>
<td>X</td>
</tr>
</tbody>
</table>
Figure 2.1 (A) Transport chamber designed to enable one-way diffusion (X direction) of solutes into cartilage half disks entering from the superficial zone (B) A slice (100-200 μm thick) was cut from the center of the cartilage half disk (6mm diameter, 1mm thick) and imaged via confocal microscopy at 10X magnification in the X-Y plane of the slice to assess penetration and X-directed solute concentration profile after a selected duration of solute transport. Red arrows indicate the direction of solute diffusion through the superficial zone (SZ). Images of the middle region of the slice (shown by dotted boundary) were taken to avoid any edge effects from the top and bottom of the half disk. A FITC (fluorescein isothiocyanate) filter cube was used for imaging FITC conjugated solutes. Quantum dots (QDs) were imaged using a filter cube containing 565/30 nm filter for green QDs, 640/50 nm filter for red QDs and a 625 nm LP dichroic mirror.

Figure 2.2 Confocal images of the concentration profile inside bovine cartilage explants of (A) FITC (MW 389 Da, diam ~0.9 nm) (B) FITC-dextran (MW 8 kDa, diam ~4.3 nm) and (C) FITC-dextran (MW 40 kDa, diam ~10 nm) after diffusion into cartilage for 24 h. (D) Confocal images of the concentration profile of FITC-dextran (MW 40 kDa, diam ~10 nm) inside cartilage explants after diffusion for 24 h, (E) 48 h, and (F) 96 h. (G) Confocal images of the concentration profile of NeutrAvidin (neutral charge, MW 60 kDa, diam ~7 nm) after diffusion into cartilage explants for 24 h, (H) 48 h, and (I) 96 h. Arrows on the right side of the images indicate cartilage superficial zone. Average fluorescence intensities across the thickness of each slice (X direction) are plotted below the images as a function of distance from the left edge of the images. Scale bar = 200 μm.
Figure 2.3 Confocal images of the concentration profile inside normal cartilage explants of 15 nm in diameter, non-functionalized (RED) Cd-Se QDs after (A) 24 h absorption, and (B) 24 h desorption (into 1X PBS bath). (C) Confocal images of the concentration profile inside normal cartilage explants of 15 nm amine functionalized QDs (GREEN) after 24 h absorption, and (D) 24 h desorption (into 1X PBS bath). (E) Confocal images of the concentration profile of 15 nm non-functionalized QDs (RED) inside trypsin treated cartilage explants (1mg/ml, 24 h treatment) after 24 h absorption, and (F) 24 h desorption (into 1X PBS bath). (G) 24 h absorption, and (H) 24 h desorption (into 1X PBS bath) of amine functionalized QDs (GREEN). Arrows on the right side of the images indicate cartilage superficial zone. Scale bar = 200 μm. (I) The percent of the initial moles of cadmium in the bath of Cd-Se red and green QDs that were absorbed into normal and trypsin treated bovine cartilage explants in 24 h. (J) The percent of cadmium absorbed in 24 h that was retained inside the cartilage explants after 24 h desorption into 1X PBS for red and green QDs and into 10X PBS for green QDs only. The explants were completely digested using HNO₃, and the total cadmium was detected using inductively coupled plasma mass spectrometry (ICP-MS). Data are for both normal and trypsin treated cartilage. Values are Mean ± SEM, n= 6 cartilage samples in each condition. Horizontal lines over bars represent significant differences between treatment groups; * indicates significant difference between trypsin treated and normal cartilage; p<0.05.
Figure 2.4 (A) Transport chamber of Fig. 1A showing visual evidence of significantly higher uptake for Avidin compared to NeutrAvidin over a 24 h period. ‘Av’ = Avidin; ‘Nu’ = NeutrAvidin. (B) Confocal images of the concentration profile inside normal cartilage explants of Avidin (positive charge, MW 66 kDa, diam~7 nm), and (C) NeutrAvidin after 24 h absorption into normal cartilage explants. Confocal images after 24 h desorption of Avidin into (D) 1X PBS and (E) 10X PBS. Arrows on the right side of the images indicate cartilage superficial zone. Scale bar = 200 μm.
Figure 2.5 Uptake ratios measured for (A) Avidin and (B) NeutrAvidin after 1 to 4 day equilibration periods for normal and 40% GAG-depleted cartilage explants (via chondroitinase-ABC) (C) The percent of moles absorbed in 24 h that was retained inside the explants after desorption into 1X and 10X PBS for Avidin and NeutrAvidin. Values are Mean ±SEM; n= 18 cartilage samples (6 disks from each of 3 animals) per treatment group for normal cartilage condition, and n=6 cartilage samples per treatment group (from 1 animal) for 40% GAG-depleted cartilage. Horizontal lines over bars represent statistical significant differences between treatment groups; * indicates significant difference between GAG-depleted and normal cartilage; p<0.05.
Figure 2.6 Concentration dependent uptake ratio of FITC labeled Avidin in cartilage explants after 3 day equilibration at 37°C in 1X PBS supplemented with protease inhibitors. Graded amounts of unlabeled Avidin was added to a fixed amount of FITC-Avidin (<1 μM). The theoretical curve of Equation (5) (solid line) was fit to the data to obtain best-fit values of $K$, $K_{EQ}$, and $N_T$. The predicted values are $K$=6, $K_{EQ}$= 150 μM and $N_T$= 2920 μM. Total bath Avidin concentration is the sum of labeled and unlabeled Avidin. Data are mean ± SD, n = 4 disks per condition.

Figure 2.7 Non-equilibrium diffusive transport of Avidin-FITC across a group of three 6 mm diameter, 400 μm thick cartilage explants, plotted as the measured downstream concentration versus time, normalized to the applied upstream concentration. At $t = 0$ min, Avidin-FITC was added to the upstream chamber. The effective diffusivity was calculated from $\tau_{lag}$ and the steady state diffusivity from the measured diffusive flux of Avidin-FITC (i.e., the slope of the concentration versus time). At $t = 186$ min, free FITC was added to the upstream chamber to estimate the contribution of unbound FITC to the total flux.
2.7. Supplementary Material

S1. Competitive binding of fluorescently labeled Avidin to intra-tissue sites in cartilage

![Graph](image)

**Figure 2S.1** Binding isotherm of Eq. (2) is re-plotted with the x-axis normalized to \( K_{\text{EQ}} \) using the best fit parameter values data of Fig. 2.6 (\( K \approx 6, K_{\text{EQ}} \approx 150 \mu\text{M} \) and \( N_{\text{T}} \approx 3000 \mu\text{M} \))

S2. Net charge of Avidin estimated from amino acid sequence

Net charge is estimated by summing over the basic (lysine, arginine) and acidic (glutamic and aspartic acid) groups which can ionize at pH 7:

<table>
<thead>
<tr>
<th>Basic Groups per chain</th>
<th>Acidic Groups per chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>+9</td>
</tr>
<tr>
<td>Arginine</td>
<td>+8</td>
</tr>
<tr>
<td>Total positive charge</td>
<td>+17</td>
</tr>
<tr>
<td>Total charge per chain</td>
<td>+5</td>
</tr>
<tr>
<td>Tetrameric structure of Avidin ( \Rightarrow +5 \times 4 = +20 )</td>
<td></td>
</tr>
<tr>
<td>Glutamic</td>
<td>-7</td>
</tr>
<tr>
<td>Aspartic</td>
<td>-5</td>
</tr>
<tr>
<td>Total negative charge</td>
<td>-12</td>
</tr>
</tbody>
</table>

However, it is not certain whether the effective charge during transport and uptake would include all residues, or whether certain residues may be internal and less accessible to charge-charge interactions
with the cartilage extracellular matrix. In addition, the unknown extent of Avidin glycosylation may contribute additional net negative charge that would decrease the total net charge.

S3. Net charge of Avidin estimated using Donnan Equilibrium:

With cartilage explants in equilibrium in a bath containing PBS and Avidin, Donnan equilibrium partitioning predicts the distribution of Avidin with charge $z$ to be related to the concentration of $\text{Na}^+$ and $\text{Cl}^-$ inside the cartilage tissue:

$$\left( \frac{\bar{C}_{\text{Avidin}}}{K_{\text{NeutrAvidin}} \bar{C}_{\text{Avidin}}} \right)^{\frac{1}{2}} = \frac{\bar{C}_{\text{Na}}}{\bar{C}_{\text{Na}}} = \frac{\bar{C}_{\text{Cl}}}{\bar{C}_{\text{Cl}}}$$

(1)

where $\bar{C}_{\text{Na}}, \bar{C}_{\text{Cl}}$ and $\bar{C}_{\text{Avidin}}$ are the intra-tissue concentrations of $\text{Na}^+$, $\text{Cl}^-$ and free (unbound) Avidin, respectively. $\bar{C}_{\text{Avidin}}, \bar{C}_{\text{Na}}$ and $\bar{C}_{\text{Cl}}$ are bath concentrations of Avidin, $\text{Na}^+$ and $\text{Cl}^-$. The results of Figs. 2.5B and 2.6 give the partition coefficients for Avidin and NeutrAvidin:

$$\frac{\bar{C}_{\text{Avidin}}}{\bar{C}_{\text{Avidin}}} = K_{\text{Avidin}} \sim 6, \ K_{\text{NeutrAvidin}} \sim 0.44.$$  

Since the bath was 1X PBS (i.e., physiological ionic strength), $\bar{C}_{\text{Na}}$ and $\bar{C}_{\text{Cl}} \sim 0.15 \text{ M}$. Also, bulk electroneutrality requires that the sum of all the charges inside the tissue is equal to zero:

$$\rho + F(\bar{C}_{\text{Na}} - \bar{C}_{\text{Cl}} + z\bar{C}_{\text{Avidin}}) = 0$$

(2)

where $\rho$ is the fixed charge density of cartilage and $F$ the Faraday constant. Assuming for the purpose of this discussion that Avidin is a minority carrier ($\sim \mu\text{M}$) compared to $\text{Na}^+$ and $\text{Cl}^-$, we neglect the contribution of $\bar{C}_{\text{Avidin}}$ in Eq. (2),

$$\frac{\rho}{F} + \frac{\bar{C}_{\text{Na}} - 0.15^z}{\bar{C}_{\text{Na}}} = 0$$

(3)

The fixed charge density for our femoropatellar groove cartilage from 1-2 week old bovine calves, $\frac{\rho}{F}$ was measured to be $-0.13 \text{ M}$ [39]. Rearranging Eq. (3) gives:

$$z = \frac{\log \left( \frac{\bar{C}_{\text{Avidin}}}{K_{\text{NeutrAvidin}} \bar{C}_{\text{Avidin}}} \right)}{\log \left( \frac{\bar{C}_{\text{Na}}}{\bar{C}_{\text{Na}}} \right)}$$

The effective net charge, $z$, for Avidin was thereby computed to be $\sim +6.2$. 

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S4. Net charge of Avidin estimated from reported electrokinetic zeta potential:

The zeta potential of Avidin molecules in dilute solution of DI water at pH 5 has been reported to be 10 mV [41]. Using Grahame Equation of electrokinetics [40] that related zeta potential to particle surface charge density, and assuming that Avidin attains a spherical shape (diameter ~ 7nm), the zeta potential of 10 mV corresponds to an effective net molecular charge of ~ +7.3.

S5. Upper bound estimate of Avidin charge assuming that Avidin uptake in Fig. 2.6 is due to electrostatic interactions (Donnan theory) and not binding:

As described in S3, Donnan equilibrium partitioning predicts the distribution of Avidin with charge z to be related to the concentration of Na⁺ and Cl⁻ inside the cartilage tissue as:

$$\left( \frac{\bar{C}_{\text{Avidin}}}{K_{\text{NeutrAvidin}}C_{\text{Avidin Bath-final}}} \right)^{1/2} = \frac{\bar{C}_{\text{Na}}}{C_{\text{Na}}} = \frac{\bar{C}_{\text{Cl}}}{C_{\text{Cl}}} = r$$

$$\rightarrow \bar{C}_{\text{Avidin}} = r^z K_{\text{NeutrAvidin}} C_{\text{Avidin Bath-final}} = r^z K_{\text{NeutrAvidin}} f C_{\text{Avidin Bath-initial}}$$

(4)

where

$$f = \frac{C_{\text{Avidin Bath-final}}}{C_{\text{Avidin Bath-initial}}}$$

(See Eqs. (3 - 4) of the main text)

It is assumed here that the uptake of Avidin was entirely due to Donnan partitioning into cartilage in the complete absence of binding to intra-tissue sites, i.e., all of Avidin inside the cartilage after equilibration is free. Avidin's uptake is defined as $R_u = K_{\text{NeutrAvidin}} r^z$.

Since the bath was 1X PBS (i.e., physiological ionic strength), $C_{\text{Na}}$ and $C_{\text{Cl}} \sim 0.15$ M, hence

$$\bar{C}_{\text{Cl}} = \frac{0.15^z}{C_{\text{Na}}} \text{ and } \bar{C}_{\text{Na}} = 0.15r$$

(5)

Bulk electro-neutrality requires that the sum of all the charges inside the tissue is equal to zero:

$$\rho + F(\bar{C}_{\text{Na}} - \bar{C}_{\text{Cl}} + z\bar{C}_{\text{Avidin}}) = 0$$

(6)

Using the measured value of $\frac{\rho}{F}$ as −0.13 and $K_{\text{NeutrAvidin}}$ as 0.44 from S2, rearranging Eqs. (4-6) gives:

$$-0.13 + 0.15r - \frac{0.15}{r} + 0.44z r^z f C_{\text{Avidin Bath-initial}} = 0$$

(7)
We varied $z$ and for different values of $C_{AvidinBath-initial}$ and calculated $r$ (without neglecting $C_{Avidin}$). The best fit was obtained for $z$ between $+13$ and $+14$ as depicted in the graph below. Avidin's uptake, $R_u$ ($K_{NeutrAvidin} r^z$) is plotted on the y-axis and $C_{AvidinBath-final}$ on the x-axis.

Figure 28.2 Upper bound estimate of Avidin charge assuming that Avidin uptake in Figure 2.6 is due to electrostatic interactions only and no binding. Best fit obtained for $z$ is between $+13$ and $+14$. 
Chapter 3  Charge Based Drug Delivery Mechanisms and a Mathematic Model of Joint Space Transport Kinetics

3.1. Introduction

In this chapter, based on our understanding of particle size and surface charge on its transport in charged tissues like cartilage, we will discuss the distinct advantages of using electrostatic interactions for local intratissue drug delivery. We will also present three methods by which particle based delivery systems can be applied to i.a. applications and finally present the governing equations to mathematically model the transport kinetics within the joint space.

3.2. Charge based drug delivery mechanisms

Electrostatic interactions provide the following distinctive advantages for transport of cationic drug carrying particles in to the negatively charged cartilage:

**High upward Donnan partitioning:** The positive charge of carriers can significantly increase their partitioning at the synovial fluid-tissue interface, owing to Donnan/Boltzmann partitioning. This will result in steep intra-tissue concentration gradient, thereby significantly enhancing the intra-tissue transport of positive drug carriers into negatively charged cartilage.

**Weak and reversible binding:** While the choice of a positively charged drug carrier does not guarantee binding to cartilage ECM, identification of cationic carriers that bind with the negative ECM via electrostatic interactions can have distinct advantages. Non-specific electrostatic interactions are most likely weak interactions which cannot strongly bind cationic drugs or drug carrying particles within the extracellular matrix of tissues. Consequently, current research in i.a. delivery has focused on using stronger binding mechanisms such as hydrogen or covalent bonds to increase the residence time of carriers inside the synovial fluid. However, strong binding mechanisms can dramatically slow the diffusion-reaction kinetics of drug carriers and prevent them from penetrating deeper into the tissue where the majority of drug targets reside. In contrast, weak and reversible electrostatic binding provides a distinctive advantage by allowing drug carriers to penetrate through the full thickness of tissues like cartilage. Appropriate cationic particles introduced inside the joint space will be attracted to and can then bind with negatively charged GAGs inside the cartilage. This weak and reversible ionic binding has a
concomitantly high dissociation constant, \( K_D \); i.e. the carriers can rapidly unbind after initial binding with their intra-tissue binding sites. If the particle size is small enough such that they are not sterically hindered by the dense meshwork of collagen and aggrecans in the ECM, they will continue to diffuse through the cartilage and penetrate deeper into the tissue.

**High intra-tissue binding site density:** The high negative fixed charge density (FCD) of cartilage provides a high density of binding sites for the positively selected charged solutes which might be expected to increase their intra-tissue residence time despite the weak binding (FCD of human cartilage \(-150 \text{mM}\)). For example, as we discussed in Chapter 2, Avidin (66,000 Da, net charge +20, pI=10.5) partitioned up by a factor of 6 inside the cartilage and remained bound within the tissue for extended time periods owing to very large intra-tissue binding site density (\( N_T = 2,920 \text{µM} \)) even though the binding was weak (\( K_D = 150 \text{µM} \)). It is important to note that partitioning and binding are two independent mechanisms that affect solute uptake (at equilibrium) and transient diffusion in very different ways. For example, Byun et al showed that while a small cationic peptide therapeutic (Arg-Tyr-Lys-Arg-Thr, 760 Da, net charge +3, pI =11) partitioned upward inside the cartilage (as expected) by a factor of 2, it did not bind within the tissue and thus diffused out rapidly [1]. Garcia et al showed that insulin like growth factor-1, IGF-1 (7,650 Da, net charge +5, pI 8.5) partitioned upward by a factor of 1.4, and bound reversibly but strongly (\( K_D \approx 5 \text{nM} \)) with known IGF-binding proteins (IGFBPs, \( N_T \approx 50 \text{nM} \)) found within cartilage ECM [2]. When IGF was further modified via addition of a heparin-binding (HB) domain to the C-terminus of IGF-1 to form the fusion protein, HB-IGF (12 kDa, net charge +13, pI 11), Miller et al. found that HB-IGF bound with high affinity to negatively charged chondroitin sulfate GAGs (\( K_D \approx 160 \text{nM} \)) due to non-specific electrostatic interactions and most likely additional specific interactions associated with the peptide sequence of the HB domain, which is known to bind heparin sulfate GAG chains. (Miller et al. hypothesized that the C-terminal location of the HB domain prevented HB-IGF-1 from binding with IGFBPs, though that hypothesis remains to be tested [3]).

**Optimal positive charge range:** If binding is purely electrostatic (as in case of Avidin), an optimal net positive charge on the particle would enable reversible binding, weak enough for the particle to diffuse into the tissue but strong enough for it to have a long residence time. Consider that the intra-tissue binding site density of the negatively charged groups is relatively large. For a given particle size, a low net positive charge implies lower probability of binding with the fixed negatively charged groups. This in turn implies a high dissociation constant, \( K_D \), and thus higher effective diffusivity (\( D_{\text{EFF}} \), diffusivity in presence of binding). Therefore, the particles will have a low residence time within the tissue. For example, zero charge on particle means \( K_D \) approaching the limit of infinity, thus \( D_{\text{EFF}} \) will become equal
to $D_{SS}$ (as $D_{EFF} = D_{SS} (1 + \frac{N_r}{K_D})^{-1}$ for the case of first order, bimolecular reversible binding). If this same particle has a very high net positive charge, it means higher probability of binding with the constant binding site density of the same tissue. This implies lower $K_D$ and thus lower $D_{EFF}$. Therefore, such particles will have a long residence time. A very low $D_{EFF}$, however, will significantly slow diffusion kinetics and prevent the particles from penetrating into the tissue. Hence there seems to exist an optimal range of positive charge on the particle that will enable rapid in-depth penetration but ensure a reasonably long residence time.

The transport of charged solutes through charged substrates (tissues) is therefore a complex process; while Donnan partitioning significantly increases concentration gradients and the resulting rate of solute influx, binding can reduce the effective diffusivity through the tissue as described above. This results in tissue becoming ‘selectively permeable’ to similarly sized solutes with varying net charge. In summary, transport of cationic drug carriers inside the tissue is governed by (i) the upward Donnan partitioning at the synovial fluid-tissue interface, (ii) the steep intra-tissue concentration gradient of drug carriers, (iii) increasing concentration of negatively charged groups (binding site density) with increasing depth into the tissue, and (iv) the convective flow of synovial fluid due to dynamic compression of the joint in addition to the particle size and net positive charge of the drug carriers.

Three methods for delivering drugs locally into cartilage via i.a. injected drug carrying particles are shown in Fig. 3.1. In Fig 3.1A, positively charged nano-sized carriers with hydrodynamic diameter less than 10 nm are chosen to minimize steric hindrance from the dense ECM of cartilage. Carriers with similar diameter but longer in length (such as a rod/chain structure) may also penetrate into the tissue owing to their tortuosity [4]. As discussed above, the positive charge of carriers significantly increases their partitioning at the synovial fluid-tissue interface resulting in steep intra-tissue concentration gradient. This enhanced intra-tissue transport of drug carriers would reach the therapeutic drug level in the shortest amount of time ($\tau_I$) compared to the other two methods described below. The weak and reversible binding allows full depth penetration. The total concentration of drug (bound plus released from the carrier) will have a profile inside the tissue similar to that of the carrier (denoted by blue line), assuming that the kinetics of release from the carrier is long enough.

The second method (Fig. 3.1B) shows a combination of small and large sized carriers functionalized to bind with cartilage ECM sites at or near the cartilage surface. Their diffusion kinetics will thereby be limited by surface binding, which will prevent them from penetrating deeper into the tissue (shown in blue). As these particles degrade, drug release will enable transport of the drug into the
cartilage, closer to tissue target sites (shown in red). Depending on its physical and chemical properties, the drug can then reach intra-tissue therapeutic levels in time \( T_2 \) such that \( T_2 > T_1 \).

The third method (Fig. 3.1C) shows large impenetrable particles without any active binding agent, and thus the particles remain suspended freely in the synovial fluid. The drug released into the synovial fluid is further away from intra-tissue target sites; thus, a significant fraction of released drug may be cleared from the joint (e.g., via capillaries and lymph) before entering cartilage or other desired tissues. The available drug concentration inside the tissue, \( C' \) will be less than the injected concentration of drug (encapsulated in carriers), \( C \). The time required to reach intra-tissue therapeutic levels (denoted by *), \( T_3 \), will be longer than \( T_2 \) such that \( T_3 > T_2 > T_1 \). This third method, however, is still better than directly injecting soluble drug into the joint synovial fluid, which would result in an even shorter residence time. Most of the currently explored “sustained release” drug delivery systems fall into the third category, e.g., the PLGA microspheres of Flexion, Inc. [5], Elastin like Polypeptides (ELP) [6] and the cationic polymeric particles cross-linked with hyaluronate developed by Bend Research [7]. A combination of methods may be the most efficient, depending on the drug properties, location of its target sites and the required residence time for effective therapy.

3.2. Joint space transport kinetics

Fig. 3.2A shows solute kinetics in the joint space after i.a. injection \((t=0)\), which instantaneously increases the synovia fluid (SF) concentration, \( C_{SF} (t = 0) = C_o \). Let’s assume a simple diffusive model inside SF with volume \( V \), with no convection or electrical migration flux. After i.a. injection of drug carriers, an orthopedist flexes the joint in an attempt to uniformly distribute the drug inside the joint. Furthermore, as the diffusivity of drug carriers within the synovial fluid is much greater than that inside the cartilage or the synovial membrane, both of which sterically hinder the transport, we can assume that the solute concentration is approximately uniform in SF at all times (and that SF is a homogenous and isotropic medium), i.e. \( C_{SF} (t) \) at SF-cartilage, SF-synovial membrane interfaces is also the same at any given point along the interfaces at any instant in time. Another reasonable simplification is to reduce the model to one-dimensional transport as shown in the scheme of Fig. 3.2B.

**Diffusive flux of entry into the cartilage:** at the synovial fluid-cartilage interface \((X = L_c)\), \( C_{SF} (t) \) will drop from \( C_o \) at \( t = 0 \) as the solute will either diffuse into the tissue or get cleared out from the membrane. \( L_c \) is the average cartilage thickness. Bone is impermeable, thus at the cartilage-bone interface \((X = 0)\),
the boundary condition is \( \frac{\partial C_c(x=0,t)}{\partial x} = 0 \) at all times. \( C_c(x,t) \) denotes concentration profile inside the cartilage. Thus,

\[
Q_{\text{Entry}} = -D_c \frac{\partial C_c(x=L_c,t)}{\partial x}, \text{ where } D_c \text{ is the effective solute diffusivity inside cartilage}
\]

Diffusive flux of exit from the synovial membrane: The lymphatics are located in the subsynovium. Thus this sink condition at the lymphatics (\( X=L_c+L_s+L_m \)) is defined by the boundary condition \( C_m(t) = 0 \) at all times, where \( C_m(x,t) \) denoted concentration profile inside the membrane. \( L_m \) and \( L_s \) denote the mean thickness of the membrane and the compartment containing SF.

\[
Q_{\text{Exit}} = -D_m \frac{\partial C_m(x = L_c + L_s, t)}{\partial x}
\]

where \( D_m \) is the effective solute diffusivity inside synovial membrane, After \( \Delta t \), the total mass of drug carriers inside the synovial fluid will depend on \( Q_{\text{Entry}} \) and \( Q_{\text{Exit}} \) as follows:

\[
V C_{\text{SF}}(t + \Delta t) = V C_{\text{SF}}(t) + Q_{\text{Exit}} A_m \Delta t + Q_{\text{Entry}} A_c \Delta t,
\]

where \( A_m \) and \( A_c \) are the surface areas of synovial membrane and cartilage respectively exposed to synovial fluid for solute diffusion. This equation can be written as:

\[
\lim_{\Delta t \to 0} \frac{(C_{\text{SF}}(t+\Delta t) - C_{\text{SF}}(t))}{\Delta t} = -\frac{1}{V} \left( D_m A_m \frac{\partial C_m(x=L_c+L_s,t)}{\partial x} + D_c A_c \frac{\partial C_c(x=L_c,t)}{\partial x} \right)
\]

\[
\rightarrow \frac{\partial C_{\text{SF}}(t)}{\partial t} + \frac{D_m A_m}{V} \frac{\partial C_m(x=L_c+L_s,t)}{\partial x} + \frac{D_c A_c}{V} \frac{\partial C_c(x=L_c,t)}{\partial x} = 0,
\]

(1)

where rate of change of concentrations inside the cartilage and membrane are defined by,

\[
\frac{\partial C_c}{\partial t} = D_c \frac{\partial^2 C_c}{\partial x^2} \text{ and } \frac{\partial C_m}{\partial t} = D_m \frac{\partial^2 C_m}{\partial x^2}
\]

(2, 3)

Initial condition, at \( t=0 \)

1. \( C_c(x < L_c) = 0 \),
2. \( C_c(x = L_c) = C_0 = C_{SF}(t = 0) \)

Boundary conditions,
(1) \( C_c(x = L_c) = C_{SF}(t) \)

(2) \( \frac{\partial C_c(x=0,t)}{\partial x} = 0 \)

(3) \( C_m(x = L_c + L_s) = C_{SF}(t) \) and

(4) \( C_m(x = L_c + L_s + L_m) = 0 \)

Equations 1-3 and the above stated boundary and initial conditions are presented to broadly define the transport processes involved. Importantly, diffusion into and/or out of the cartilage tissue itself is hereby included specifically; to our knowledge, this aspect has not been highlighted as such before. A solution of this system of equations can be obtained through a computational model and used to determine drug carrier concentration in the SF, \( C_{SF} \), as a function of time, and to characterize the total residence time of particles inside the joint, \( \tau_{SF} \). This residence time (\( \tau_{SF} \)) will depend on the length scales, surface areas, and volumes of the cartilage, synovial fluid and the synovial membrane as well as the effective diffusivities of particles in them. Scaling analysis of equations (2-3) shows that the time for drug-carriers to transport in and out of cartilage, \( \tau_c \propto \frac{L^2}{D} \). The exact solution, however, will depend on the boundary conditions.

**Fig. 3.2B** shows schematic representations of the transient solute concentration profiles in the synovial membrane, SF and cartilage. The average \( C_{SF}(t) \) will decrease with time as the particle concentration increases inside cartilage \( (C_c(t)) \) and particles clear out from the joint space via the synovium. When the particle concentration in cartilage and SF becomes equal (i.e., upon achieving pseudo equilibrium), there will begin a net outward diffusion of drug carrying particles from the cartilage back to the SF in the absence of any binding mechanism. This time required for particles to transport in and out of cartilage, \( \tau_c \), can be calculated from the model. Likewise the total residence time of particles inside the joint, \( \tau_{SF} \), can be estimated. We can also determine the time required to reach the drug therapeutic level as well as the total time, \( \tau_{eff} \) for which the drug stays above this critical level (denoted by *) inside the cartilage. This time, \( \tau_{eff} \), is the one of interest as during this time, the drug would be effective in eliciting a biological response.

### 3.3. Summary

In this chapter, we have proposed three methods of how particle based delivery systems can be used for local drug delivery into cartilage and also discussed their pros and cons. We have presented an analysis of the effects of increasing net positive charge on cationic particles on their diffusion kinetics and retention inside cartilage. We have also presented the governing equations to model transport kinetics.
inside the joint, which can be used for developing a computational model as a tool for initial screening of various drug carrying particles for different applications.

3.4. References


Increasing effectiveness of particle based DDS

Increasing rate of achieving drug therapeutic threshold

A
+ Charged penetrable particles

Rapid & full depth penetration
'Electrostatic interactions'
\( k = \text{Donnan Partitioning} \)
Drug conc profile is similar to carrier conc profile (blue)

B
Sticky large/small particles

Surface adsorption of drug carriers
'Binding limited kinetics'
Large carriers sterically hindered
Drug conc profile shown in red (dotted line)

C
Impenetrable particles

No penetration of drug carriers
'No binding to cartilage ECM'
Carriers suspended in SF
Drug released gets cleared out
thus \( C' < C \)

\( T_1 < T_2 < T_3 \)

C is the concentration of drug (encapsulated in carriers) in the synovial fluid. \( C' \) is the effective drug concentration in SF after clearance from the joint. Blue curves show concentration gradient of drug carriers. Red dotted curves show concentration gradient of the drug released from these carriers inside the cartilage. \( * \) denotes the drug therapeutic threshold. \( T \) represents time to achieve therapeutic threshold

**Figure 3.1** Three classes of particle based drug delivery systems for local i.a. intra-tissue delivery of drugs
Figure 3.2 (A) Kinetics of i.a. injected drug carrying particles inside the joint space (B) Concentration profiles
Chapter 4 Electrostatic interactions enable rapid penetration, enhanced uptake and retention of intra-articular injected Avidin in rat knee joints*

Intra-articular (i.a.) drug delivery for local treatment of osteoarthritis remains inadequate due to rapid clearance by the vasculature or lymphatics. Local therapy targeting articular cartilage is further complicated by its dense meshwork of collagen and negatively charged proteoglycans, which can prevent even nano-sized solutes from entering. In a previous in vitro study, we showed that Avidin, due to its size (7nm diameter) and high positive charge (pl 10.5), penetrated the full thickness of bovine cartilage and was retained for 15 days. With the goal of using Avidin as a nano-carrier for cartilage drug delivery, we investigated its transport properties within rat knee joints. Avidin penetrated the full thickness of articular cartilage within 6h, with a half-life of 29h, and stayed inside the joint for 7 days after i.a. injection. The highest concentration of Avidin was found in cartilage, the least in patellar tendon and none in the femoral bone; in contrast, negligible Neutravidin (neutral counterpart of Avidin) was present in cartilage after 24h. A positive correlation between tissue sGAG content and Avidin uptake ($R^2=0.83$) confirmed the effects of electrostatic interactions. Avidin doses up to at least 1 μM did not affect bovine cartilage explant cell viability, matrix catabolism or biosynthesis.

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4.1. Introduction

Osteoarthritis (OA) affects individual joints, necessitating localized therapy [1,2]. Intra-articular (i.a.) injections allow for local and targeted delivery of drugs into the joint space, thereby reducing systemic toxicity. However, i.a. therapy often remains inadequate due to rapid clearance of drugs from the joint space; small molecules exit via the vasculature while larger macromolecules (e.g., hyaluronan) are cleared by the lymphatic system [2,3]. Mean half-lives of NSAIDs in the synovial fluid have been reported to be 1-4 h [4,5]. Solutes in synovial fluid with sizes similar to plasma proteins (albumin ~ 67kDa, globulin ~ 150 kDa, fibrinogen ~ 340 kDa) have equal permeability through the lymphatics[2]. Their clearance, however, is dependent on the rate of synovial fluid turnover and solute diffusivity; the latter is a function of solute size and molecular weight, viscosity of synovial fluid and temperature. For example, intra-articular half-lives in normal rabbit knee joints have been reported to range from 0.23 h for Acridine Blue (370 Da) to 1.23 h for Albumin (67 kDa) and 26.3 h for Hyaluronan (300 kDa) [2,3].

Intra-articular injection of drug-encapsulating particles can increase half-lives of therapeutic drugs in the synovial fluid [1,6,7]. However, therapeutic efficacy depends on the ability of the drugs (or particle-bound drugs) to penetrate into specific target tissues and to be retained by those tissues over time. Entry of macromolecules into cartilage is hindered by its dense extracellular matrix (ECM) of collagen fibrils and aggrecan proteoglycans containing highly negatively charged glycosaminoglycan (GAG) chains [8]. Hence, it is critical to develop appropriate particle based i.a. delivery methods that can (1) facilitate a faster rate of drug/particle transport into target tissues compared to their rate of clearance from vasculature or lymphatics, and (2) enable penetration and binding of drugs within target tissues for sustained intra-tissue depot delivery to cell/matrix targets, thereby minimizing the need for multiple injections that deliver much higher doses to synovial fluid than needed at the cell/matrix target.

Transport of solutes is size and shape dependent, while binding within cartilage ECM is modulated by particle surface properties, including charge. We showed that solutes having a hydrodynamic diameter <10 nm penetrated the full thickness of bovine cartilage explants, while 15 nm diameter particles were sterically hindered and trapped in the superficial zone[8]. The high negative fixed charge density of cartilage offers the unique opportunity to utilize electrostatic interactions for augmenting solute transport, binding and retention. We used Avidin, a globular 66kDa (diameter ~ 7 nm) positively charged protein (pI 10.5), as a model for charge driven transport. Avidin penetrated the full thickness of bovine cartilage within 1 day, while its neutral same-sized counterpart, Neutravidin, penetrated only half the sample thickness in 4 days. Avidin had a 400-fold higher uptake than
Neutravidin, and was bound to intra-cartilage GAGs due to reversible, weak electrostatic interactions. High uptake was demonstrated even after enzymatic removal of ~40% of tissue GAG, mimicking the GAG loss in early OA [8]. These attributes of Avidin structure are especially suitable for particle based drug delivery to cartilage.

With the goal of using Avidin as a drug delivery carrier, we injected fluorescently labeled Avidin into the knee joints of healthy rats, investigating avidin uptake kinetics, distribution and retention in the joint tissues over 7 days. Using bovine cartilage explants in vitro, we also studied the effects of Avidin on chondrocyte viability, sGAG content and matrix biosynthesis in order to determine safe limits for Avidin-functionalized drug delivery.

4.2. Methods

4.2.1. In-vivo study design: Animal studies were performed as pre-approved by the Institutional Animal Care and Use Committee at BIDMC. Injections of 50 µl of 50 µM Avidin or Neutravidin conjugated with Texas Red (both from Invitrogen, CA) were administered through the patellar tendon into the right knee joints of healthy, 18-20 week old Fischer-344 rats (Charles River Laboratories). Following injection, the knee was flexed and extended to distribute the injected solute throughout the intra-articular space. Contralateral left knees were used as controls. Avidin injected rats were sacrificed at 4 different time points (6h, 1d, 4d, 7d) and Neutravidin injected rats were sacrificed at 1 day. The following tissues were extracted from each knee joint of treated and control animals: articular cartilage from the patellofemoral groove, femoral condyle, tibial plateau and patella using a scalpel (together analyzed as ‘cartilage’); medial and lateral menisci; anterior and posterior cruciate ligaments (ACL and PCL); patellar and quadriceps tendons; and femoral bone. We used 6 rats per treatment condition, for a total of 30 rats. Using a digital caliper, the medial-lateral joint width was measured before injection and afterwards at each time point to check for joint swelling as an indication of an inflammatory response to the injection.

4.2.2. Confocal Microscopy: Tissue samples extracted from the rat joints injected with Avidin-Texas Red and explanted at 6 h were imaged using confocal microscopy (OLYMPUS, FluoView FV1000) at 10x magnification. Z stacks were taken in the X-Y plane, which represents the surface into which Avidin diffused. 3D images were reconstructed from the Z stacks, and cross-sections in the X-Z plane of these 3D images were taken. Confocal images of tissue specimens from the contralateral control knees exhibited no fluorescence.
4.2.3. **Quantitative analysis of Avidin/Neutravidin uptake into tissue samples:** Samples from all conditions were desorbed in 10x PBS for 48h at 37°C to disrupt electrostatic interactions and release Avidin/Neutravidin into the desorption bath. Desorption for > 48 h did not increase the fluorescence signal in the bath. Samples were removed from the bath, lyophilized and the dry weight measured. Fluorescence signals from the desorption bath were quantified using a plate reader (Synergy HT, BioTek). In establishing standard curves, fluorescence intensities were linear with bath concentrations of both Avidin-Red and Neutravidin-Red. Solute uptake was calculated as the concentration of labeled solute in the tissue normalized to tissue dry weight.

4.2.4. **sGAG measurement and histological analysis:** The sGAG content of each tissue was measured using the dimethyl-methylene blue (DMMB) dye binding assay [9] following digestion with proteinase K. For histology, whole naïve rat knee joints or joint tissues harvested from naïve rat knees were simultaneously fixed and decalcified in Formical-4 (Decal Chemical Corporation) for 24-72 h, depending on the bone content of the tissue specimen. Following decalcification, specimens were dehydrated and embedded in paraffin, and either sagittal or coronal sections (5 μm) were made at 300 μm intervals throughout the specimen. Sections were stained with Toluidine Blue to image the amount and distribution of sGAG, staining all sections simultaneously to minimize variation in staining among specimens.

4.2.5. **Effects of Avidin on bovine cartilage in-vitro:** Bovine cartilage disks were harvested from the patellofemoral grooves of 1-2 week old bovine calf knees (Research 87, Boylston, MA) as described. Briefly, cylindrical disks (3 mm diam) were cored using a dermal punch and sliced to obtain the top 1 mm of cartilage with intact superficial zone. Disks for all treatment groups were matched for location along the joint surface. Disks were pre-equilibrated for 48 h in serum free low glucose DMEM (1g/L) supplemented with 10 mM HEPES buffer, 0.1 mM nonessential amino acids, 0.4 mM proline, 20 g/ml ascorbic acid, 100 units/ml penicillin G, 100g/ml streptomycin and 0.25 g/ml amphotericin B (all Sigma Aldrich, MO) in a 5% CO₂ incubator at 37°C. Groups of disks were cultured for another 48 h with Avidin doses of 0, 100nM, 1μM or 100 μM. Medium was then changed every 2 days without replenishing Avidin for a total culture duration of 2, 4 or 10 days, thereby simulating a single Avidin injection in vivo.

4.2.6. **Chondrocyte viability:** Upon termination of culture at each time point, 100-200 μm thick slices were cut from the centers of the bovine disks for each treatment condition. These slices were immediately stained for 2-3 minutes in the dark with fluorescein diacetate (FDA; 4mg/ml) and propidium iodide (PI; 40mg/ml) in PBS (both from Sigma Aldrich, MO). FDA stained viable cells green, while PI stained non-
viable cells red. The slices were then washed with PBS and imaged using a fluorescence microscope (Nikon Eclipse Ti-S) with a 4x objective.

4.2.7. sGAG loss to medium and chondrocyte protein and sGAG biosynthesis: Two days before the termination of day 4 and day 10 explant cultures, the medium was supplemented with 5 μCi/mL [³⁵S]-sulfate and 10 μCi/mL [³H]-proline (PerkinElmer, Norwalk, CT). After 48 h of radiolabeling, disks were washed 4 times over 80 minutes with cold PBS to remove free label. After measuring wet weight, disks were digested with proteinase K (Roche, Indianapolis, MN) overnight. Cumulative sGAG released to the medium and the residual sGAG in digested explants were measured using the DMMB assay. The radiolabel in each digested sample and medium standard was measured using a liquid scintillation counter (Perkin Elmer MicroBeta TriLux). Radiolabeled concentration was calculated from the standards and then normalized to the wet weight of the cartilage disks. (Avidin binding to DNA prevented its use for normalization.)

4.2.8. Statistical Analysis: Data on Avidin and sGAG concentration within rat tissue specimens are presented as Mean ± SD from 6 and 7 animals, respectively, for each treatment condition. We used the general linear mixed effects model with animal as a random variable for analysis followed by Tukey’s test to test the effects of tissue type and time on Avidin uptake, and to compare relative sGAG concentration in different tissue types. (There was no effect of animal.) Data on sGAG loss and proteoglycan and protein biosynthesis in bovine explants are derived from 2 animals with 6 explants per animal for each treatment condition; they are presented as mean ± SEM for n=12 disks per condition (the general linear model showed no effect of animal). We used p<0.05 for statistical significance. Statistical analysis was performed using Systat 12 software (Richmond, CA).

4.3. Results

4.3.1. Avidin injection into rat joints: Following i.a. injection of Avidin, rat knee joints did not present any signs of swelling or stiffness through 7 d. After sacrifice, the articular cartilage, menisci, i.a. ligaments, and patellar and quadriceps tendons were harvested to investigate the depth of penetration and retention of Avidin. Avidin-Texas Red staining in rat knee joints and extracted tissues was strikingly visible at the 6 h and 24 h time points. Figs. 4.1A and 4.1B compare staining between control and Avidin injected knees at 6 h (Texas red is dark purple to the naked eye). Confocal images showed that Avidin diffused through the full thickness of these tissues within 6 h after i.a. injection (Fig. 4.2). Contralateral control knees showed negligible fluorescence.
By 6 h after i.a. injection, the pooled mean concentration of Avidin in the different articular cartilages was \( 4.7 \pm 0.9 \mu g/mg \) tissue dry weight, which decreased to \( 3.3 \pm 0.95 \mu g/mg \) by 24 h (Fig. 4.3). Avidin concentration further decreased to 10.3% of the 24 h value (0.34 ± 0.19 \( \mu g/mg \)) at 4 days and 4.1% of 24 h value at 7 days (0.13 ± 0.09 \( \mu g/mg \)). Similar rates of reduction in Avidin concentration were observed in other tissue types over 7 days (Fig. 4.3). The half-life of Avidin was calculated by fitting an exponential to the experimental mean values: \( C(t) = C_0 \exp(-t/\tau) \), where \( C(t) \) is the concentration in the tissue at time \( t \), \( C_0 \) is the initial Avidin concentration, and \( \tau \) is the characteristic exponential decay time (\( \tau \) and the associated half-lives of Avidin are shown in Table 4.1 for each tissue type). We used Avidin’s neutral counterpart, Neutravidin, and compared its uptake into rat knee tissues with that of Avidin at the 24 h time point. There was no detectable Neutravidin present in rat cartilage and patellar tendon at 24 h, but very small amounts were present in meniscus, ligaments and quadriceps tendon compared to Avidin (Fig. 4.3). Neither Avidin nor Neutravidin was detected in femoral bone explants at any time point.

Correlation of Avidin uptake with sGAG content: sGAG concentration measured for the different rat cartilages had a pooled mean concentration of \( 18.3 \pm 5.98 \mu g/mg \) tissue wet weight, consistent with data reported by others in the literature [10-12]. The sGAG contents for quadriceps tendon (5.8 ± 1.89 \( \mu g/mg \)), ligaments (4.1 ± 1.55 \( \mu g/mg \)), menisci (3.6 ± 0.2 \( \mu g/mg \)) and patella tendon (1.5 ± 0.38 \( \mu g/mg \)) were similar to that reported previously for rabbit and sheep [13,14]. The relatively high sGAG concentration in the quadriceps tendon may be due to the presence of sesamoid fibrocartilage, called the suprapatella, embedded in the deep surface of the quadriceps tendon immediately above the patella. It has been shown previously that the rat suprapatella contains aggrecan and link protein, and the suprapatella is present in many mammals including mouse, rats, rabbits, and dogs, but is absent in humans [15,16]. The half-life of Avidin retention in different tissue types (Table 4.1) correlated positively with the respective sGAG concentration (R\(^2\)=0.83), confirming the effects of strong electrostatic interactions (Fig. 4.4B).

Toluidine Blue staining of the knee tissues (Fig. 4.5) revealed the relative concentration and spatial distribution of sGAG within different joint tissues harvested from naïve rats. A slight metachromasia (from blue to purple) specified sGAG within the articular cartilage of the femoral condyle (Fig. 4.5A-B), tibial plateau (Fig. 4.5C-D), and patellofemoral groove (Fig. 4.5E-F), as well as along the surface of the menisci (Fig 4.5C-D). More intense sGAG staining was observed in the cartilage of the epiphyseal plate as compared to the articular surfaces (Figs. 4.5A,C,E). From sections taken in the sagittal plane, the suprapatella could be distinguished from the adjacent quadriceps tendon by increased sGAG staining and the absence of parallel collagen fibers, which were stained blue-green (Fig. 4.5G-H). Weak
metachromasia was also observed within the i.a. ligaments (e.g., the ACL) near the femoral/tibial insertion sites (Fig. 4.5I-J). These observations were all consistent with DMMB assay results (Fig. 4.4A).

**4.3.2. Effect of Avidin dose on chondrocyte viability:** As a measure of the safe limit of Avidin dose, cell viability was assessed via live-dead fluorescence in bovine explants after a one time, 2-day Avidin treatment (0-100 μM) and subsequent culture without Avidin for an additional 4 or 10 days. Representative images from 3-4 cartilage disks from each treatment condition (Fig. 4.6) showed minimal cell death in no-Avidin controls over 10 days. Qualitatively, cell viability did not change markedly with time over 10 days in any treatment group. Note that some cell death in the soft superficial zone was typically observed even in untreated explants, depending on the location of harvesting along the joint surface.

**4.3.3. Cumulative sGAG loss and biosynthesis:** There was no significant difference in cumulative sGAG loss to medium between the untreated control, 100nM and 1μM Avidin treated conditions measured at 4 and 10 days (Fig. 4.7A). However, 100 μM Avidin resulted in higher sGAG loss compared to controls at 4 days (7% for control vs. 14.6% for 100 μM) and 10 days (13.6% vs. 20%). There were no significant differences in the rate of protein and sGAG synthesis for any Avidin dose compared to controls (Fig. 4.7B and 4.7C).

**4.4. Discussion**

The complex architecture of cartilage can prevent even nano-sized solutes from entering into its deeper zones, making local delivery of drugs a challenge [2]. Recent studies showed that while 8kDa (~4.3nm) dextran penetrated the full thickness of normal bovine cartilage within 24 h, it took 4 days for 40kDa (~10nm) dextran to penetrate through half the tissue thickness [8]. This problem is further complicated by rapid clearance of drugs by lymphatics or vasculature. Avidin, due to its ideal size and high positive charge, exhibited a fast rate of uptake, 400x higher than its neutral counterpart in bovine cartilage [8].

The rat studies presented here, which account for the presence of lymphatics and convective transport within the joint space, showed that Avidin penetrated throughout the full thickness of different intra-articular tissue types within 6 h, resulting in highest uptake within cartilage, least within the patellar tendon and none within the femoral bone (Fig. 4.3). A positive correlation ($R^2 = 0.83$) between the sGAG concentration in different tissues (Fig. 4.4A) and their respective Avidin half-lives (Table 4.1) confirmed the effects of strong electrostatic interactions between positively charged Avidin and the negatively
charged GAGs within these tissues. These interactions enhanced Avidin transport due to Donnan partitioning, enabling rapid penetration into joint tissues prior to clearance by the lymphatic system. Avidin was retained inside the rat joint for 7 days, with a half-life of 29 h in cartilage, whereas Neutravidin exhibited almost complete clearance within 24 h of i.a. injection. Half-lives of similar sized solutes like albumin have been reported to range between 1.23-3.9 h in the rabbit knee [3].

Several drugs have been identified as potentially useful to reverse or prevent post-traumatic osteoarthritis (PTOA) and the associated breakdown of cartilage, including anti-catabolic glucocorticoids (e.g., dexamethasone) and pro-anabolic growth factors (e.g., IGF-1, FGF-18, and BMP-7) [17-20]. However, sustained intra-tissue delivery is needed for efficacy. For example, half-lives of 1-4 h for glucocorticoids (<700 Da) have been reported in the human joint space [3], requiring the use of multiple injections. Systemic side effects are also a concern for certain drugs, requiring intra-articular delivery without subsequent clearance into the circulation. A vehicle like Avidin can potentially transport candidate drugs into cartilage at a fast rate and bind reversibly to create a drug depot inside cartilage. Avidin may be particularly useful for drug delivery in the early stages of PTOA before the proteoglycan content of cartilage has substantially diminished. In addition to cartilage, the observed uptake of Avidin in ligaments can be exploited for delivering pro-anabolic growth factors following ligament injury.

The peak concentration of Avidin in rat cartilage was 3 times lower than that reported for bovine cartilage in vitro. This may be due to the 2.5-3x lower sGAG concentration of cartilage in the rat compared to young bovine. We reported >90% retention of Avidin in bovine cartilage explants for at least 15 days [8]. Due to the presence of convective transport within the rat model, a drop in retention was expected. However, the 4.1% retention at 7 day in the rat study is more likely due to the 10x lower thickness of rat cartilage compared to bovine. The diffusion-binding time constant scales as the square of the thickness, so the transport rate into and out of rat cartilage is expected to be 100 times faster. An animal model having a thicker cartilage like rabbit or goat [10] will be a closer representation of human physiology and transport kinetics.

We also studied dose dependent biological effects of Avidin to estimate a safe range for delivery. A one-time dose of 0-100 µM Avidin did not cause any change in chondrocyte viability in bovine cartilage explants over 10 days. While sGAG loss to the medium did not change for a 0-1 µM dose range during this test period, there was an approximate 2-fold increase in sGAG loss for the 100 µM dose at both 4 and 10 days compared to untreated controls. At 100 µM, Avidin may reduce intra-tissue osmotic pressure, leading to decreased water content and slight loss of proteoglycans. However, chondrocyte biosynthesis of proteins and proteoglycans was not affected, suggesting that Avidin did not cause
chondrotoxicity. Taken together, these results suggest that Avidin doses up to at least 1 μM are safe. Recently, it was shown that a continuous dose of 100 nM dexamethasone significantly reduced the catabolic effects of mechanical injury and increased levels of inflammatory cytokines in human and bovine cartilage explants [18]. Dexamethasone can be conjugated to 4 binding sites within Avidin [21], providing at least a 4:1 molar ratio of dexamethasone to Avidin. Thus <1 μM doses of Avidin are estimated to be able to provide a sustained depot delivery of 100 nM dexamethasone.

In summary, the in vivo rat data presented here further support the use of electrostatic interactions for augmenting transport and increasing retention of Avidin or Avidin-like nanoparticles into cartilage due to its size and high positive charge. Since Avidin can be conjugated with a variety of therapeutics, there is the potential for safe and effective intra-articular delivery of such therapeutics.

4.5. References


Table and figures

Table 4.1: Mean lifetime and half-lives of Avidin retention in rat knee tissues.

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>$\tau$ (mean lifetime, hours)</th>
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<td>Cartilage</td>
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<tr>
<td>Meniscus</td>
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<td>21.4</td>
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<td>Ligament</td>
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A. 6h Av i.a. injection

B. 6h Contralateral control

Figure 4.1 (A) Images of rat knee joint following injection with Texas Red-conjugated Avidin, showing visual evidence of high Avidin uptake within different joint tissues at 6 h. The purple color of Texas Red is visible throughout the joint space (marked by circles). (B) Images of a contralateral control knee joint are shown for comparison.
Figure 4.2 Confocal images (10x magnification) of tissue specimens extracted from rat knee joints 6h after intra-articular injection of Avidin-Texas Red (excitation at 595 nm; emission at 615 nm). Images were taken in the X-Y plane, which represents the superficial surface (SS) through which Avidin diffused. 3D images were reconstructed using Z stacking (slice thickness ~4.5 μm). Cross-sections in the X-Z plane of these 3D stacks are shown.
Figure 4.3 Avidin (Av) and Neutravidin (Nu) uptake and retention in different tissue types from rat knee joints after 6 h, 24 h, 4 days and 7 days (Av) or after 24 h (Nu). Each treatment condition represents tissue specimens from the right knee joints of six rats. Tissue specimens from contralateral (left) knee joints were used as controls for each treatment condition and showed negligible fluorescence. Data are presented as Mean+/-SD.
Figure 4.4 (A) sGAG concentration measured using DMMB assay in different rat knee tissues, expressed as µg sGAG per mg wet weight of tissue. Data is presented as Mean±SD, N= 7 animals. * represents statistical significance compared to articular cartilage (p<0.05). (B) sGAG concentration vs. Avidin half-lives for different tissue types (C-articular cartilage; QT- Quadriceps Tendon; L-Ligaments (ACL & PCL); M-Menisci; PT-Patellar Tendon). Diamonds represent experimental data, solid line is the linear least squared fit and dotted lines show 95% confidence intervals. $R^2 = 0.8316$. The 95% confidence interval lines have positive slope, indicating that trends are significant.
Figure 4.5 Toluidine blue staining of naïve rat knee tissues, sectioned in either the coronal (A-B) or sagittal (C-J) plane. Images were taken at 2.5x magnification (A, C, E, G, and I) and, for the regions of interest denoted by insets, 10x magnification (B, D, F, H and J). (A-B) Medial femoral condyle with underlying epiphyseal plate. (C-D) Medial joint compartment showing tibial plateau and meniscus. (E-F) Femoral trochlea and patellar tendon. (G-H) Quadriceps tendon and adjacent suprapatella along with proximal portion of patellar bone and cartilage. (I-J) Anterior cruciate ligament with tibial and femoral attachments. ACL-anterior cruciate ligament, AHC-articular hyaline cartilage, AHMM-anterior horn of the medial meniscus, BM-bone marrow, EP-epiphyseal plate, FB-femoral bone, HFP-Hoffa’s fat pad, PB-patellar bone, PT-patellar tendon, QT-quadriceps tendon, SP-suprapatella, SY-synovium, TB-tibial bone.
Figure 4.6 Images of fluorescently stained bovine cartilage explants (4x objective) after treatment with increasing dose of Avidin (0, 100nM, 1μM and 100μM) for 2, 4 and 10 days to check for chondrocyte viability: 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 = 200 μm.
Figure 4.7 (A) Cumulative sGAG loss from bovine cartilage to the culture medium after 4 and 10 days treatment with a one time dose of Avidin (0, 100nM, 1µM or 100µM for 2 days). (B) Bovine chondrocyte protein synthesis and (C) sGAG synthesis during the last 48 h of culture were measured using $^3$H and $^{35}$S incorporation rates, respectively, for the same cartilage disks as in (A). Values are Mean+/-SEM; N= 2 animals (6 disks/animal). * represents statistical significance compared to control untreated condition (p<0.05).
Chapter 5 On the choice of animal model for intra-articular drug delivery relevant to treatment of post-traumatic osteoarthritis*

For evaluation of new approaches to drug delivery into cartilage, the choice of an animal model is critically important. Since cartilage thickness varies with animal size, different levels of drug uptake, transport and retention should be expected. Simple intra-articular injection can require very high drug doses to achieve a concentration gradient high enough for drug diffusion into cartilage. New approaches involve nanoparticle delivery of functionalized drugs directly into cartilage; however, diffusion-binding kinetics proceeds as the square of cartilage thickness. In this study, we demonstrate the necessity of using larger animals for sustained intra-cartilage delivery and retention, exemplified by intra-articular injection of Avidin (drug-carrier) into rabbits and compared to rats in vivo. Penetration and retention of Avidin within cartilage is greatly enhanced by electrostatic interactions. Medial tibial cartilage was the thickest of rabbit cartilages, which generated the longest intra-cartilage half-life of Avidin ($\tau_{1/2} = 154$ h). In contrast, Avidin half-life in thinner rat cartilage was 5-6 times shorter ($\tau_{1/2} \sim 29$ h). While a weak correlation ($R^2 = 0.43$) was found between Avidin half-lives and rabbit tissue GAG concentrations, this correlation improved dramatically ($R^2 = 0.96$) when normalized to the square of cartilage thickness, consistent with the importance of cartilage thickness to evaluation of drug delivery and retention.

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5.1. Introduction

The choice of animal model is extremely critical for understanding osteoarthritis (OA) pathophysiology, for the discovery of new OA biomarkers and for the testing of novel disease modifying therapies [1-3]. Many potential OA therapies have shown beneficial effects in animals but have failed in human clinical trials [4-6]. The translatability of preclinical research is largely dependent on the anatomical and biomechanical similarities between the animal models used and humans [2,7]. Small animals have commonly been used in OA research due to their lower costs and ease of manipulation relative to larger species; more recently, the development of transgenic mouse models have broadened their utility [2,3,8]. While rodent models are powerful tools for investigating biological disease mechanisms and initial screening of therapeutics, data from larger animal models are clinically more relevant and generally preferred by the FDA [2,3]. Here, we focus on additional engineering criteria associated with the importance of larger animal models for delivery and retention of drugs within cartilage relevant to OA/PTOA.

Simple intra-articular (i.a.) injection can require very high drug doses to achieve a concentration gradient high enough for drug diffusion into cartilage. Such high doses can cause deleterious effects; even low-dose sustained release in the synovial fluid may still cause unwanted drug exposure to other joint tissues. New approaches involve delivery of functionalized drugs directly into cartilage using micro and nanoparticles [9-14], drug releasing peptides and gels [15-16]. Small animal models have been widely used for their evaluation [9-13]. The transport kinetics of drugs or drug carrying vehicles within the joint space is not only dependent on their size/molecular weight and surface functional properties but also the biophysical properties of the animal joint and its constituent tissues. Both the size of the joint space and the thickness of soft tissues (cartilage, menisci, ligaments, tendons) increase with overall animal size [17,18]. For example, the following mean thickness values of articular cartilage for various mature animal species have been reported: mouse ~50 μm, rat ~100-150 μm, rabbit ~350-700 μm, goat ~900 μm, pig ~1.5 mm, horse ~1.5-2 mm and human ~1.5-2 mm [17-21].

In this study, we demonstrate the significance of using animals larger than rodents for investigation of tissue-targeted intra articular (i.a.) drug delivery. As an example, we use Avidin, which has been shown to possess ideal characteristics of a drug carrier for i.a. delivery into cartilage due to its optimal size and high positive charge [22].
Fluorescently labeled Avidin was i.a. injected into the knee joints of healthy 12 month old female rabbits, and its transport kinetics and retention in various knee tissues were compared with a similar study conducted in rats [23]. We report 5-6 times longer half-lives of Avidin in rabbit cartilage than in rat cartilage, which can be attributed to the difference in cartilage thickness since the kinetics of diffusion-reaction transport depends on the square of cartilage thickness. Our results demonstrate the critical importance of cartilage thickness and thus animal size to assess techniques for cartilage drug delivery.

5.2. Methods

5.2.1. In-vivo study design: Animal studies were performed as pre-approved by the Institutional Animal Care and Use Committee at BIDMC (this is a pre-clinical study). Injections of 300 µl of 75 µM Avidin conjugated with Texas red (Invitrogen, CA) were administered through the patellar tendon into the right knee joints of 12 healthy, 12 month old female New Zealand white rabbits (average weight 5kg). Following injection, the knees were flexed and extended several times to distribute the injected solute throughout the intra-articular space. Avidin injected rabbits were sacrificed at 3 different time points (days 1, 4, and 7). In 3 of the 4 animals used at each time point, the contralateral left knees were injected with saline as vehicle controls; in the 4th animal, Avidin was also injected into the left knee. Thus, 5 joints were injected with Avidin-red for each of the three time points from a total of 12 rabbits in the study. The following tissues were extracted from each knee joint: tibial articular cartilage from lateral (LTC) and medial (MTC) sides; femoral cartilage from both lateral (LFC) and medial (MFC) sides; patella and trochlear groove cartilage (PC and GC); lateral and medial menisci (LM and MM); anterior and posterior cruciate ligaments (ACL and PCL), patellar tendon (PT), quadriceps tendon (QT) and long digital extensor tendon (ET). A scalpel was used to remove full thickness articular cartilage from the underlying bone. Due to the low volumes of tissue harvested from the patella and groove, they were pooled together (PC+GC) for analysis. Using a digital caliper, the medial-lateral joint width was measured at the mid-length of the patellar tendon before and after injection at each time point to check for joint swelling as an indication of any inflammatory response to the injection.

5.2.2. Quantitative analysis of Avidin uptake into tissue samples: Extracted tissue samples from all conditions and time points were first weighed wet, then desorbed in 10x PBS for 72h at 37°C to disrupt electrostatic interactions [22] and release Avidin into the desorption bath. (Desorption for greater than 72h did not increase the fluorescence signal of the bath.) Samples were removed from the bath, lyophilized and weighed dry; the water weight was calculated from tissue wet and dry weights. Fluorescence signals from the desorption bath were quantified using a plate reader (Synergy HT, BioTek).
In establishing standard curves, fluorescence intensities were linear with bath concentrations of Avidin-red. Avidin uptake (concentration in the tissue) was calculated as the amount of labeled Avidin desorbed from the tissue normalized to tissue water weight.

5.2.3. sGAG measurement and histological analysis: The sGAG content of each tissue sample was measured using the dimethyl-methylene blue (DMMB) dye binding assay [24] following digestion with proteinase K. For histology, tissues from one contralateral control were harvested, fixed and decalcified in Formical-4 (Decal Chemical Corporation) for 24-72 h, depending on the bone content of the tissue specimen. Following decalcification, specimens were dehydrated, embedded in paraffin, and either sagittal or coronal sections (5 μm) were made at 300 μm intervals throughout the specimen. Sections were stained with Safranin-O to image the amount and distribution of sGAG, staining all sections simultaneously to minimize variation in staining among specimens.

5.2.4. Measurement of tissue thickness: Cartilage thickness was measured from Safranin-O stained histology images as previously described [18]. A closed boundary from the articular surface to the cartilage-bone interface was identified from the Safranin-O stained cartilage area. Thickness was estimated by drawing a random number of lines perpendicular to the boundaries delineated by the articular surface and the tidemark. An average of the lengths of these lines was taken as an estimate of the mean cartilage thickness, and the standard deviation was calculated as a measure of variation in the tissue thickness across the sectioning plane. As a secondary check of this method, we also used a needle probe method [20] to measure thickness of selected lateral tibial plateau cartilage samples. The needle was mounted in the load cell assembly of a Dynastat mechanical spectrometer (IMASS, Hingham, MA); the joint was mounted in a special fixture with a ball joint assembly which allowed easy movement to position cartilage surface perpendicular to the needle. The needle was ramped into the cartilage at 20 μm/s and the load was continuously recorded. Location based thickness was calculated by using the time recorded between the needle contacting first the articular surface and then the calcified zone of the cartilage. These readings were within 10% of that estimated using the histology images.

5.2.5. Statistical analysis: Data are represented as mean values ± SD or 95% confidence intervals, as shown. Linear least squares regression is used to compare sGAG content versus intra-cartilage half-life of Avidin across different rabbit knee tissue types, with and without normalization to the square of cartilage thickness.

5.3. Results
5.3.1. Avidin injection into rabbit joints: The i.a. injection of Avidin conjugated with Texas red was well tolerated by the rabbits. Their knee joints did not present any signs of swelling or stiffness. Figs. 5.1A-B compare staining between control (saline injected) and Avidin injected knees at 24 h. Avidin-red staining (Texas red is dark purple to the naked eye) was well distributed throughout the knee joint and, using previously described methods [23], penetrated thoroughly within tissues including articular cartilage, the articulating surface of the quadriceps tendon, and the inner rim of the menisci (Fig 5.1B). However, in two cases during i.a. delivery, the needle accidentally hit the anterior cruciate ligament (ACL). In those cases, much of the Avidin was unintentionally delivered locally to the ACL, as can be seen from the locally concentrated staining of Avidin-red in ligaments of Fig. 5.1B.

The retention of Avidin measured by end of 1 day, 4 days and 7 days after i.a. injection was markedly different between the various joint tissue types (Fig. 5.2). The contralateral control knees of all animals showed no fluorescence, suggesting that there was no systemic crosstalk during the 7-day duration of the experiments. The half-life of Avidin retained in the injected knees was estimated by fitting an exponential to the experimental mean values: \( C(t) = C_0 \exp(-t/\tau) \), where \( C(t) \) is the concentration in the tissue at time \( t \), \( C_0 \) is the initial Avidin concentration, and \( \tau \) is the characteristic exponential decay time (\( \tau \) and the associated half-lives, \( \tau_{1/2} \), of Avidin are shown in Table 5.1 for each tissue type).

5.3.2. Thickness assessed from histology: Safranin O staining of a saline-injected contralateral knee revealed strong GAG staining within the articular cartilage of the femoral condyles, tibial plateau, patella, and trochlear groove (Fig. 5.3A-F). As was observed in the rat knee [23], the articulating surface of the rabbit quadriceps tendon contains a GAG-rich structure known as the suprapatella (Fig. 5.3G). This structure accounts for the considerable Avidin-red uptake seen within the quadriceps tendon (Fig. 5.1B). Using histology images, the mean thicknesses of articular cartilage from tibia, femur, patella and trochlear groove were measured and are reported in Table 5.2. These results are similar to that reported previously for mature rabbit cartilage tissues (LTC ~500µm and MTC ~740µm [19,20]; LFC ~310-455µm and MFC ~350-470µm [17,18]).

5.3.3. sGAG content: The sGAG concentration measured for the different rabbit cartilages (Fig. 5.4A) was 2-3 times higher than that recently reported for rat cartilages [23]. We found the lowest concentration of sGAG to be in cartilages combined from the patella and trochlear groove, PC+GC (27.4 ± 11.5 µg/mg, mean ± SD) and the highest in the medial femoral cartilage, MFC (47.4 ± 3.7 µg/mg). Interestingly, the sGAG concentrations for the quadriceps tendon (5.2 ± 2.8 µg/mg), ligaments (6.4 ±1.3
μg/mg), patella tendon (1.1 ± 0.34 μg/mg) and the long digital extensor tendon, ET (3.1 ± 1.0 μg/mg) were similar to that reported previously for rat [23], while sGAG in the menisci (LM: 11.8 ± 7.3 μg/mg, MM: 8.6 ± 3.4 μg/mg) were 2.5-3 times higher than that found in rat.

5.3.4. Correlation of Avidin uptake with tissue sGAG content: We previously reported a strong correlation (R² = 0.83) between the half-lives of Avidin retention in different rat tissue types (i.e., cartilages, ligaments, tendons and menisci) and their sGAG concentrations. This result in vivo [23] confirmed the findings with bovine cartilage explants in vitro [22] that strong electrostatic interactions between positively charged Avidin and negatively charged sGAGs greatly increased uptake of Avidin preferentially into cartilages. Here, we similarly report the half-lives of Avidin retention in different rabbit tissue types (Table 5.1) and, based on their respective sGAG concentration (Fig 5.4A), we found a weak correlation between Avidin and sGAG content, R² = 0.43 (Fig 5.4B). There are two important observations to note: (1) The lateral and medial femoral cartilages (LFC and MFC) had the highest sGAG concentration and also resulted in the highest mean uptake values of Avidin at 24h (LFC: 1.9 ± 1.2 μg/mg; MFC: 1.4 ± 0.6 μg/mg) but not the longest half-lives (τ₁/₂ for LFC = 60.7h and for MFC = 70.2h). (2) The medial tibia cartilage, MTC, had a lower Avidin uptake (0.6 ± 0.05 μg/mg) at 24h than the femoral cartilages but retained Avidin for the longest period of time (τ₁/₂ = 153.9h). We measured MTC to be the thickest of all of the other articular cartilage tissues, consistent with the data reported by others [19,20].

Sources of error:

The large error bars in Fig. 5.2 are associated with the accidental delivery of Avidin into anterior cruciate ligament in two rabbits. In such cases, Avidin was not well distributed throughout the joint space resulting in lower uptake values in other tissue types compared to the case of normal i.a. delivery. Thus, while the patellar tendon approach is recommended to ensure joint space delivery in smaller rat knees (i.e., maximum injection volume ~50 μL), an anterolateral infrapatellar approach through the joint capsule is recommended for i.a. injection into the rabbit knee.

5.4. Discussion

For the evaluation of new approaches for drug delivery to articular cartilage, the choice of animal model for investigation is important. Since cartilage thickness varies by the size of animal [17], different levels of drug uptake, diffusion-reaction transport kinetics and retention within cartilage should be expected. While drug carrying particles may be able to penetrate into the thickness of mouse or rat tissues
quickly because of their small thickness, such particles may be cleared out from the joint space of larger animals before achieving a similar extent of intra-tissue penetration. In the case of Avidin, its transport kinetics within the joint space and uptake and binding within cartilage are dominated by electrostatic interactions. It has been shown that a high Donnan partitioning of Avidin into cartilage drives its rapid rate of transport and uptake inside cartilage [22] (an upward partition factor of ~6 was measured in bovine cartilage).

Once a drug reaches desired therapeutic levels inside cartilage (with no blood supply), the theoretical retention time (i.e., the outward diffusion-reaction time constant, $\tau_{dr}$) scales as square of the tissue thickness ($\delta$) as given by $\tau_{dr} \propto \left( \frac{\delta^2}{D_{EFF}} \right)$, where $D_{EFF}$ is the effective diffusivity of the solute within the tissue including the effects of binding reactions [25,26]. The thickness of rabbit articular cartilage can be 2-10 times larger than that of rat cartilage depending on location [17-19,23] and hence the rate of transport out of rabbit cartilage is expected to be 4-100 times slower. Our data confirm this, as we measured the longest half-life of Avidin in the medial tibia cartilage (MTC) of rabbit as 153.9h compared to rat cartilage, where Avidin half-life was found to be 5-6 times shorter (half-life ~ 29h) [23]. Due to the small tissue volume harvested from rat joints, articular cartilage was combined from different surfaces (tibial plateau, femoral condyle, patella and trochlear groove) and analyzed together as ‘cartilage’. In a previously published in-vitro study using 1mm thick young bovine cartilage explants, we reported >90% retention of Avidin for at least 15 days [22].

In vivo, a decrease in retention time is expected (compared to in vitro explants) due to the presence of convective transport and clearance by the lymphatic system. By day 7 in the rat model, Avidin retention was only 4.1% of the initial value at 24h after i.a. injection [23]. Given that rat cartilage is ~10 times thinner than the 1mm thick bovine explants, exudation transport would be ~100 times faster from the rat cartilage, consistent with our observations. Using adult rabbits with much thicker cartilage than rats (or mice), we showed a significant improvement in Avidin retention time, implying that retention in thicker human cartilage would be even greater.

We found a weak but positive correlation ($R^2 = 0.43$) between calculated Avidin half-lives and the corresponding sGAG concentration measured in different rabbit tissue types (Fig. 5.4B). This correlation improved greatly (to $R^2 = 0.96$) when Avidin half-lives were re-plotted versus sGAG density normalized to the square of tissue thickness (Fig. 5.5), underlining the significance of incorporating tissue thickness as a parameter when considering drug delivery and retention within cartilage.
Since sGAG concentration does not change dramatically with animal size [17,18], the time to reach intra-cartilage therapeutic levels of, e.g., Avidin-conjugated drugs, would not be that different between species. However, retention time is a function of not only binding within the tissue extracellular matrix but also the square of tissue thickness. Hence, conjugation of desired drugs with Avidin (or Avidin-like solutes) would result in longer half-lives in the cartilages of larger animals as well as in humans.

5.5. References


Tables and figures

Table 5.1: Mean lifetime and half-lives of Avidin retention in rabbit knee tissues

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<th>Tissue Type</th>
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<td>Medial Tibial Cartilage (MTC)</td>
<td>153.9</td>
<td>222.0</td>
</tr>
<tr>
<td>Patella &amp; Groove Cartilage (PC+GC)</td>
<td>96.3</td>
<td>139.0</td>
</tr>
<tr>
<td>Medial Femoral Cartilage (MFC)</td>
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<td>101.3</td>
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<tr>
<td>Lateral Femoral Cartilage (LFC)</td>
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<td>87.6</td>
</tr>
<tr>
<td>Quadriceps Tendon (QT)</td>
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<td>87.1</td>
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<tr>
<td>Lateral Tibial Cartilage (LTC)</td>
<td>56.8</td>
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<tr>
<td>ACL &amp; PCL</td>
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<td>Lateral Meniscus (LM)</td>
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<td>61.9</td>
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<tr>
<td>Long Digital Extensor Tendon (ET)</td>
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<td>58.0</td>
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<tr>
<td>Medial Meniscus (MM)</td>
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<td>55.5</td>
</tr>
<tr>
<td>Patellar Tendon (PT)</td>
<td>21.0</td>
<td>30.3</td>
</tr>
</tbody>
</table>

Table 5.2: Mean thickness of articular cartilage measured using histology images

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Articular cartilage thickness in ( \mu m ) (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lateral Femoral Cartilage (LFC)</td>
<td>274 ± 61</td>
</tr>
<tr>
<td>Lateral Tibial Cartilage (LTC)</td>
<td>440 ± 74</td>
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<tr>
<td>Medial Femoral Cartilage (MFC)</td>
<td>295 ± 60</td>
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<tr>
<td>Medial Tibial Cartilage (MTC)</td>
<td>740 ± 45</td>
</tr>
<tr>
<td>Patellar Cartilage (PC)</td>
<td>710 ± 73</td>
</tr>
<tr>
<td>Groove Cartilage (GC)</td>
<td>305 ± 45</td>
</tr>
<tr>
<td>Mean thickness used for analysis of pooled Patellar &amp; Groove Cartilage (PC+GC)</td>
<td>600*</td>
</tr>
</tbody>
</table>

*Mean thickness is calculated based on the individual weight contribution of GC and PC to the pooled weight of GC+PC.
Figure 5.1 (A) Images of tissues harvested from the rabbit’s contralateral control knee joint. (B) Images of knee joint following injection with Texas Red-conjugated Avidin, showing visual evidence of high Avidin uptake within different joint tissues at 1 day. The purple color of Texas Red is visible throughout the joint space. PC: Patellar cartilage; QT: Quadriceps tendon; PT: Patellar tendon; ACL & PCL: Anterior & posterior cruciate ligaments.
Figure 5.2 Avidin uptake and retention in different tissue types from rabbit knee joints after 1 day, 4 days and 7 days. Each treatment condition represents tissue specimens from 5 knee joints of 4 rabbits. Tissue specimens from contralateral (left) knee joints were used as controls and showed no fluorescence. Data are presented as Mean ± SD.
Figure 5.3 Safranin-O staining of joint tissues harvested from the rabbit knee from the contralateral control group, sectioned in either the coronal (A, C, F) or sagittal (B, D, E, G) plane. Images A-E, G were taken at 5x magnification and F at 10x. (A) Lateral femoral condyle. (B) Lateral tibial plateau. (C) Medial femoral condyle. (D) Medial tibial plateau. (E) Patellar cartilage. (F) Trochlear groove. (G) Quadriceps Tendon (QT) with adjacent suprapatella (SP). Scale bar is 200 μm.
Figure 5.4 (A) sGAG concentration measured using DMMB assay in different rabbit knee tissues, expressed as µg sGAG per mg wet weight of tissue. Data is presented as Mean±/SD, N= 5 joints from 4 animals. (B) sGAG concentration vs. Avidin half-lives for different tissue types. Diamonds represent experimental data, solid line is the linear least squared fit and dashed lines show 95% confidence intervals. \( R^2 = 0.42 \).
Figure 5.5 Avidin half-lives for different tissue types plotted against as a function of sGAG concentration* tissue thickness square. Diamonds represent experimental data, solid line is the linear least squared fit and dashed lines show 95% confidence intervals. $R^2 = 0.96$. 

$R^2 = 0.958$
Chapter 6  Targeted intra-tissue sustained delivery of
dexamethasone using Avidin as a nano-carrier for treating
degenerated cartilage*

Intra-tissue sustained delivery of disease modifying osteoarthritis drugs is necessary as majority target sites reside inside the deep zones of articular cartilage. The dense extracellular matrix and the high density of negatively charged proteoglycans make cartilage impenetrable to larger sized drugs or drug carrying particles. Avidin, owing to its small-enough size and optimal positive charge, has been shown to rapidly penetrate through full thickness cartilage and bind within the tissue resulting in long half-lives in vivo. In this work, we use Avidin as the nano-carrier for delivering dexamethasone (DEX) in a cytokine induced cartilage injury model in-vitro. Avidin is conjugated with DEX using a combination of two chemical linkers: fast drug releasing ester linker and a slow drug releasing-pH sensitive hydrazone linker. Their drug release profiles are characterized and their bioactivity is tested in cartilage organ culture explant systems. A single dose of Avidin-DEX at the start of culture, successfully suppressed cytokine induced catabolic effects over 3 weeks in this living cartilage culture system. A single dose of soluble DEX, however, had a shorter lived biological response and thus needed to be replenished continuously throughout the culture period. Glucocorticoids like DEX are small molecules and rapidly clear out from the joint space requiring multiple injections of high drug doses. Our data suggests that a single intra-articular injection of Avidin-conjugated drug can enable sustained drug delivery in low doses and therefore has the potential to replace the current clinical practice of using multiple injections of high dose glucocorticoids in patients.

* This chapter is a manuscript in preparation for submission as follows:

AG Bajpayee, MA Quadir, PT Hammond, AJ Grodzinsky, “Targeted intra-tissue sustained delivery of dexamethasone using Avidin as a nano-carrier for treating degenerated cartilage”
6.1. Introduction

Biologically, OA is characterized by increased synovial fluid concentrations of pro-inflammatory cytokines such as IL-1, IL-6 and TNFα, which can diffuse into the cartilage and initiate proteolysis and loss of cartilage matrix [1-3]. Until now, OA therapy has primarily focused on symptomatic treatment. Several disease modifying drugs with the potential to inhibit or even reverse the progression of OA have been discovered and are in experimental phase. Ineffective local delivery methods, however, have necessitated the use of high drug doses, which cause negative side effects like bone desorption and systemic organ toxicity [4-6]. As such many OA clinical trials have failed [7] and no OA drug has been brought to practice. Since pain is the main reason for patients with OA to seek medical help, clinical practice and the pharmaceutical industry are now focusing on the development of new approaches for more effective and longer lasting pain management [8-10]. With pain being the current regulatory primary end point, such drugs can be tested in shorter clinical trials and thus are attractive candidates for commercialization. However, pain relief does not indicate slowing down of OA progression. In fact, as drugs relieve pain, patients can be more susceptible to overloading their joints thereby accelerating the progression to late stage OA, a problem encountered during anti-NGF clinical trials [11]. Thus an effective OA treatment will require drug carrying cartilage penetrable nanoparticles that can enable sustained release of small doses of drugs inside cartilage to inhibit the catabolic effects associated with OA progression, while providing pain relief to joint tissues.

With the goal of achieving local, targeted drug delivery to cartilage, we conjugated Avidin with dexamethasone (DEX), used here as an example small molecule drug. DEX has been shown in-vitro to suppress cytokine induced catabolic activities via GC receptor-dependent pathways [12], and, when used with anabolic factors (e.g., IGF-1) to maintain matrix biosynthesis levels. Avidin has been shown to have no adverse effect on safety in humans [13,14] as well as no effect on cartilage viability or biosynthesis even at high concentrations in-vitro [15]. As covalent attachment of poly(ethylene glycol) (PEG) can shield the potential immunogenic responses [16] to highly cationic species, we conjugated PEG to Avidin to ensure in-vivo compatibility within the joint. Initially, DEX was supramolecularly encapsulated within native and PEGylated Avidin to construct a nanoscale, Avidin based drug delivery vehicle (abbreviated as Av+DEX and PEG Av+DEX respectively). In addition, to attain controlled release of DEX from such a nanoparticle delivery construct, we attached the drug to biotinylated PEG through ester (for fast drug release) or hydrazone (for slower and sustained release) linkages, which in turn were supramolecularly coupled to Avidin through the Avidin-biotin interaction.
Drug release profiles from the two covalently modified constructs (PEG Av-ester-DEX and PEG Av-hydrazone-DEX) were compared with their non-covalently constructed counterparts. The biological activity of these constructs was then tested using an in vitro model of cartilage catabolic injury incorporating IL-1α treated cartilage explants in live organ culture.

6.2. Materials and Methods

The chemical structures and schematics of the four configurations of Avidin-DEX conjugates are shown in Fig 6.1. All chemical compounds were purchased from Sigma Aldrich, MO unless specified.

6.2.1. Supra-molecular entrapment of Avidin with 3H-DEX (non-covalent conjugation)

Compound A (Av+DEX, Fig 6.1) was synthesized by dissolving Avidin in 1x PBS and adding to the solution an amount of unlabeled DEX equivalent to two times the weight of Avidin used, and an amount of 3H-DEX (specific radioactivity: 100 Ci/mmol, concentration: 1mCi/mL, American Radiolabeled Chemicals, MO) equivalent to yield a final concentration of 5µCi/mL.

For synthesizing compound B (PEG Av+DEX, Fig 6.1), Avidin was PEGylated utilizing its 4 biotin binding sites by mixing 4 molar equivalents of biotinylated PEG-amine (2.3 kDa) with 1 molar equivalent of Avidin (Invitrogen, CA) in 1x PBS at room temperature for 2.5h. The fluorescent probe 2,6 ANS (Invitrogen, CA) dye assay was used to confirm the stoichiometric binding of biotinylated PEG to Avidin as previously described [17]. Conjugation of biotinylated PEG to Avidin will affect its relative electrophoretic mobility [18] and this was confirmed by 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 using Coomassie brilliant blue. Similar to compound A, we added an amount of unlabeled DEX equivalent to two times the weight of Avidin used, and an amount of 3H-DEX (specific activity: 100 Ci/mmol, concentration: 1mCi/mL) to yield a final concentration of 5µCi/mL.

The solutions (both PEGylated and native Avidin versions) were stirred overnight at r.t. The solutions were then filtered using 0.2 µm mesh filters followed by ultra-filtration using a 3.5kDa mesh and spinning at 8,000g for 30 minutes to remove any free DEX from the solution that was not functionalized to Avidin. The concentration of DEX in the final solutions was estimated by measuring the radioactivity using a liquid scintillation counter (microBeta TriLux, Perkin Elmer). Avidin concentration was determined by using the bicinchoninic acid (BCA) assay [19]. The drug loading content (DLC) in the final solutions was estimated as follows:
**6.2.2. Synthesis of compound C (PEG Av-ester-DEX)**

Compound C (Fig 6.1) was synthesized by a two-step process. In the first step, Dex was conjugated to biotinylated PEG using an *ester linker* (3 of Scheme 1). In the second step, biotinylated PEG-DEX conjugate was supramolecularly attached to Avidin.

**Step 1A: Synthesis of dexamethasone hemisuccinate:**

DEX was succinylated to form dexamethasone hemisuccinate by completely dissolving 0.030 g of DEX (1 of Scheme 1, 0.076 mmol, 1.0 equiv.) in 1 mL of pyridine (non-anhydrous) following addition of 0.038 g of succinic anhydride (0.382 mmol, 5.0 equiv.) as shown in Scheme 1 according to a previously published procedure [20]. For incorporation of $^3$H-DEX, a fixed amount of $^3$H-DEX (0.2 nmol, specific activity: 100 Ci/mmol) was added as a tracer. This solution was used as radiolabel standard for calculating DEX concentration in the final product. Then 1-2 mg of DMAP (7.6 μmol, 0.1 equiv.) was added to the solution and reaction was allowed to run for 24h at r.t. under N$_2$-flow. After 24h, pyridine was evaporated under reduced pressure (in a rotary evaporator). A volume equivalent to 10 mL of H$_2$O was added to the evaporated residue. A white precipitate was observed, which was stirred for 10 minutes and then centrifuged. The resulting precipitate was washed again with 10 mL of H$_2$O and the washed residue was lyophilized to yield the target product (2 of Scheme 1).

**Step 1B: Conjugation of DEX hemisuccinate with biotinylated PEG-amine (2.3 kDa):**

Dexamethasone hemisuccinate from step 1A (2 of Scheme 1, 0.020 g, 0.0406 mmol, 1.0 equiv.) was completely dissolved in anhydrous DMF. An amount equivalent to 0.039 g (5.0 equiv.) of EDCI was added to the solution followed by 0.0187 g (4.0 equiv) of NHS. The reaction solution was activated for 15 minutes under nitrogen after which 0.244 g (0.048 mmol, 1.2 equiv.) of biotinylated PEG amine was added. The reaction was allowed to run for 48h under N$_2$-flow. After 48h, the solution was concentrated and passed through size exclusion chromatography with Sephadex LH20 using DMF as mobile phase. Eluted fractions containing PEGylated DEX fraction and solvent evaporated to yield viscous yellowish product of biotinylated PEG-dexamethasone (3 of Scheme 1). The structure was confirmed with $^1$H NMR spectral profile using Bruker 400 MHz spectrometer. $^1$H NMR ($d_6$-CH$_3$CN + D$_2$O) δ ppm: 8.5 (s), 6.87 (d), 6.66 (s), 5.15 (s), 5.10 (s), 4.16 (-CH$_2$-CH$_2$-O-, PEG protons), 3.86 (-CH$_2$-CO-NH-N=), 3.86-3.60 (b), 3.50 (s), 3.23 (s), 2.36-2.30 (b), 2.07, 1.59-1.37 (b)

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6.2.3. Synthesis of compound D (PEG Av-hydrazone-DEX)

Compound D (Fig 1) was synthesized by a two-step reaction protocol. In the first step, biotinylated PEG-DEX compound was synthesized using pH sensitive hydrazone (HZ) linker (6 of Scheme 2). In the second step, biotinylated PEG-DEX was conjugated with Avidin.

Step 1A: Synthesis of (β-maleimidopropioyl) hydrazone of Dexamethasone (4):

Compound 4 of Scheme 2 was synthesized according to the previously described procedure with modification[21,22]. DEX (1 of Scheme 2, 0.076 mmol, 1.0 equiv.) and N-β-maleimidopropionic acid hydrazide trifluoroacetate salt (BMPH) (0.228 mmol, 3.0 equiv.) were dissolved in 5 mL of methanol. For synthesis of structures labeled with ³H-DEX, a fixed amount of ³H-DEX (0.2 nmol, specific activity: 100 Ci/mmol) was added as a tracer. This solution was used as radiolabel standard for calculating DEX concentration in the final product. Trifluoroacetic acid (1.92 uL) was added and the solution was stirred at room temperature for 48h. The methanolic solution was concentrated under reduced pressure at 31 °C to a volume of 0.96 mL. To this concentrated solution, 5 mL PBS (pH 7.4) was added and the resulting precipitate was collected by centrifugation, washed with fresh PBS and lyophilized to yield (β-maleimidopropioyl) hydrazone of dexamethasone (4 of Scheme 2). The product was directly used in the conjugation step 1B with biotinylated PEG without further purification.

Step 1B: Conjugation of (β-maleimidopropioyl) hydrazone of dexamethasone to biotinylated-PEG amine (5):

Iminothiolane hydrochloride (0.002 mg, 20 μmol, 5.0 equiv.) was dissolved in 1 mL of sodium phosphate buffer with EDTA (pH 7.0). Biotinylated PEG amine (0.020 g, 4.0 μmol, 1.0 equiv.) was dissolved in 0.5 mL of the same buffer. Iminothiolane hydrochloride solution prepared before was added to the PEG solution and the reaction is allowed to stir for 20 min. (β-maleimidopropioyl) hydrazone of dexamethasone (4 of Scheme 2) was dissolved in minimum volume of DMSO and was added slowly to the buffer solution containing iminothiolane activated biotinylated PEG amine (5 of Scheme 2). The reaction was allowed to run for 1 overnight. The precipitate was centrifuged out and discarded. A short dialysis of the supernatant of 4h was carried out against PBS (pH 7.4) to remove DMSO, and the solution was lyophilized to yield the final product after passing through sephadex LH 20 column (6 of Scheme 2).

³H NMR (d₆-CH₃CN) δ ppm: 7.78 (s), 6.08 (b), 5.85 (s), 5.77 (s), 4.90-4.69 (m), 4.11 (b), 3.40 (CH₃-CH₂-O-, PEG protons), 3.06 (m), 2.91 (m), 2.82 (m), 1.79 (m), 1.68 (m), 1.38 (s), 0.97 (t), 0.90 (t), 0.70 (b), 0.69 (d).
**Step 2: Conjugation with Avidin**

Both biotinylated PEG-DEX compounds with ester and hydrazone linkers were dissolved in 1x PBS. DEX concentration in the $^3$H labeled structures solution was estimated by measuring radioactivity by using a liquid scintillation counter (Perkin Elmer MicroBeta TriLux) and calibrating it with the standards saved during synthesis. Biotinylated PEG-dexamethasone (4.0 molar equivalent) was mixed with 1 molar equivalent of Avidin in 1x PBS at room temperature for 2.5h. Fluorescent probe 2,6 ANS (Invitrogen, CA) dye assay was used to confirm biotinylated PEG-DEX to Avidin molar ratio as described before. To quantify DEX loading, conjugate was hydrolyzed with 0.1 N HCl (1 mg/ml) overnight. The resulting solution was neutralized and analyzed with reverse-phase HPLC (Agilent 1200 series, Agilent Technologies Inc, CA) using gradient elution through Discovery C18 column (Sigma Aldrich, MO) at 25 ℃. The mobile phase consisted of acetonitrile/TEAA buffer (50/50, v/v) with a flow rate of 1.0 mL/min. DEX was detected at 235 nm and calibration curve was generated by plotting the weight of DEX in µg versus the peak area in the HPLC chromatogram using a series of DEX standards. Synthesis steps for both covalent structures (ester and hydrazone linkers) are shown in Schemes 1 and 2.

**6.2.4. In-vitro drug release**

Release of DEX from Avidin conjugated DEX structures was measured by using dialysis membranes (Spectra/Por Float-A-Lyzer G2) having a molecular weight cutoff of 3,500 Da. A solution of Avidin conjugated with $^3$H labeled DEX (5 mL) was placed in a dialysis bag and immersed in a flask containing 200mL of 1x PBS at pH 7.4 or 4.0 (for hydrazone linked conjugate, pH adjusted with 1.0 N HCl) at 37℃. The flask was kept on a magnetic stirrer. At different time intervals (starting at $t=0$), aliquots of solution (50µL) were withdrawn from inside the dialysis bag and the radioactivity ($^3$H) in the solution was measured using a liquid scintillation counter to estimate the concentration of residual DEX in the dialysis bag. The percent drug release was calculated using the following equation, where $t$ is the time at which radioactivity is measured and $t_o$ is the initial time

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

**6.2.5. Biological response in bovine cartilage OA model in-vitro**

Cartilage disks (3mm x 1mm thick) with intact superficial zone were harvested from the femoropatellar grooves of 1-2 week old bovine calf knee joints (obtained from Research 87, Hopkinton, MA) as previously described [23]. 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)), 10 mM HEPES buffer (Invitrogen, CA), supplemented with 1% ITS (insulin-transferrin-selenium, at 10μg/ml, 5.5 μg/ml and 5ng/ml, respectively), 0.1 mM nonessential amino acids, 0.4 mM proline, 20 μg/mL ascorbic acid, 100 units/mL penicillin G, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (all from Sigma Aldrich, MO) for two days in a 37°C, 5% CO_2 incubator prior to any treatment.

**Treatment of living bovine cartilage explants with exogenous cytokine and soluble DEX**

To establish the baseline comparison for studying the effectiveness of our Avidin-DEX constructs, we first treated groups of cartilage explants with 1ng/mL of IL-1α for 16 days in the presence of the following DEX-treatment conditions: (i) a single dose of 100nM DEX, and (ii) a continuous dose of 100nM DEX. Medium was changed every 2 days and 1ng/mL of IL-1α was replenished at each medium change. In the ‘single DEX dose’ conditions, cartilage explants were subjected to DEX for only the first 2 days, and subsequent medium changes did not contain any DEX, thereby simulating a single i.a. injection of DEX in-vivo. Medium for the ‘continuous DEX dose’ treatments were, 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 be effective in suppressing IL-1α induced GAG loss in cartilage with only ONE dose added on day 0 of culture, bovine cartilage explants were treated either 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 100 nM, 4 μM and 100 μM). Since 4 moles of DEX are covalently conjugated using ester linker with 1 mole of Avidin, DEX concentrations of 100nM, 4 μM and 100 μM imply a theoretical Avidin loading of 25nM, 1 μM and 25 μM, respectively.

To test the biological effectiveness of Avidin-conjugated DEX compounds, we treated cartilage samples with or without 1ng/mL of IL-1α 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 (HZ) linker (PEG Av-HZ-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 alone. An effective concentration of 100μM DEX was thereby used in all treatments.

**Test of chondrocyte viability in explant culture with Avidin-DEX compounds**
Upon termination of culture, 100-200 μm thick slices were cut from the center of disks from each treatment condition using established methods [23]. The 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) (both from Sigma Aldrich, MO). FDA stained viable cells green and PI stained non-viable cells red [24]. The slices were washed with PBS and then imaged using Nikon fluorescence microscope with a 4x objective.

**Measurement of cartilage sGAG loss to medium and sGAG biosynthesis in cartilage**

Two days before the termination of organ culture of day-8 experiments, the medium was supplemented with 5 μCi/ml [35 S]-sulfate (PerkinElmer, Norwalk, CT). After 2-day radiolabel period, explants were washed 4 times over 80 minutes with cold PBS to completely remove the free label. Each individual explant was first weighed wet and then digested with proteinase K (Roche, MN) overnight. The cumulative release of sulfated GAG (sGAG) to the medium and the residual sGAG in the digested explants were measured using the dimethyl-methylene blue (DMMB) dye binding assay [25]. The amount of radiolabel in each digested sample and medium standards (35S) were measured using a liquid scintillation counter. Radiolabel concentration was calculated from the standards and was then normalized to explant wet weight.

**6.2.6. Statistical Analysis**

Data in Fig. 6.3A-C are from 4-5 DEX release experiments and are presented as Mean ± Standard Deviation. Data in Figs. 6.5 and 6.6A-C are presented as Mean ± SEM. Data in Fig. 6.5 are from 3 animals, n=6 explants per treatment condition from each animal. Data in Fig. 6.6A are presented from one animal, n=6-12 explants per treatment condition, and data in Fig. 6.6B are from 4 animals for all conditions except for the single DEX dose treatment where N= 3 animals; n=6-12 explants for each condition from each animal. Data in Fig. 6.6C are from 4 animals; n=6 explants for each condition from each animal. For all cartilage explant studies, we used the general linear mixed effects model with animal as a random variable, followed by the Tukey’s test for comparisons between multiple treatment conditions. There was no effect of animal found and, hence, the data across animals were pooled. We used p < 0.05 for statistical significance.

**6.3. Results and Discussion**

**6.3.1. Characterization of Avidin conjugated DEX structures**
As shown in Fig. 6.1 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 below:

<table>
<thead>
<tr>
<th>Compound</th>
<th>Compound type</th>
<th>DLC (wt%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Av + DEX</td>
<td>33.2 ± 4.8</td>
</tr>
<tr>
<td>B</td>
<td>PEG Av + DEX</td>
<td>32.8 ± 3.6</td>
</tr>
<tr>
<td>C</td>
<td>PEG Av-ester-DEX</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>D</td>
<td>PEG Av-hydrazone-DEX</td>
<td>1.9 ± 0.1</td>
</tr>
</tbody>
</table>

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 6.2A). 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 PEG also resulted in a shift of molecular weight (MW) as observed in SDS-PAGE from the band at ~16 kDa (Fig 6.2B, monomeric form of Avidin (Av) in lane 1 shifted to the broader band at higher MW (PEG Av in lane 2) due to the addition of 2.3 kDa PEG chains).

6.3.2. In-vitro DEX release from Avidin

In vitro release profiles of DEX from non-covalent compounds in PBS at 37°C and pH 7.4 are shown in Fig. 6.3A. Approximately 70% of the loaded DEX was released from compound A (Av+DEX) in 3h. Pegylation of Avidin (Compound B) did not significantly affect this release rate. This release rate was similar to DEX diffusivity in PBS under the same conditions indicating the absence of any robust binding interaction between DEX and the macromolecular assembly of Avidin alone, or PEGylated Avidin. In contrast, the conjugation of DEX to Avidin via ester linkages significantly slowed the release of DEX (Fig. 6.3B, 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 6.3B).
Conjugation of DEX to Avidin via hydrazone linkage (compound D) slowed DEX release even further (Fig 6.3C). The hydrazone bond undergoes only limited cleavage at pH 7.4, with maximum release at 30% loading, but the bond becomes less stable in an acidic environment [26-28]. Consistent with this behavior, DEX release from the hydrazone linker showed a mean half-life of 57.4 ± 3.6h at pH 4. The initial fast release of DEX may be attributed to non-covalently bound DEX on Avidin, which implies a longer half-life than 57.4h.

6.3.3. 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 by using a live-dead fluorescence assay. Bovine cartilage explants were treated for 48h with compounds C and D, PEG Av-ester-DEX and PEG Av-hydrazone-DEX (final DEX concentration of 100 μM). Representative images from 3-4 cartilage disks from all treatment conditions at 48h (Fig. 4B,C) showed minimal cell death and were similar to untreated controls (Fig. 6.4A). Thus, cell viability after treatment with Avidin-DEX (ester or hydrazone linker) was qualitatively similar to that in untreated control, confirming that these compounds did not cause cytotoxic effects. Note that some cell death in the superficial zone was typically observed in untreated explants, depending on the location of harvesting along the joint.

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

The inflammatory cytokine IL-1α is well known to be elevated after joint injury and is one of the family of cytokines that plays a critical role in facilitating events associated with progression to post traumatic osteoarthritis (PTOA) [29]. It was previously shown that a low concentration of 1ng/mL IL-1α induced loss of cartilage aggrecan fragments containing negatively charged sGAGs and suppressed matrix biosynthesis in young bovine cartilage in vitro [12,30]. Treatment of these samples with 10-100nM dexamethasone (DEX) continuously over 8 days significantly blocked the cytokine induced cartilage degradation [12,24] and in the presence of 1% ITS [12] or IGF-1 [24] alleviated suppressed matrix biosynthesis via glucocorticoid receptor-dependent pathways.

IL-1α treatment (1ng/ml) caused a significant increase in the loss of sGAG-containing aggrecan fragments from cartilage explants over 16 days compared to controls (Fig 6.5), which is known to result from IL-1-induced upregulation of aggrecanases (ADAMTS-4,5) by the chondrocytes in bovine [31] as well as human [3] articular cartilage. In our study, cumulative sGAG loss was 5x higher in IL-1α treated explants compared to untreated controls by day 16. Both a single dose (at day 0) and continuous doses
(starting at day 0) of 100nM DEX significantly inhibited sGAG loss until day 10 compared to IL-1α alone. However, at day 12, there was a substantial increase in sGAG loss from the single-DEX-dose explant condition, at the same rate as that from IL-1α treatment alone, while the continuous DEX dose maintained inhibition of sGAG loss through day 16. This suggests that the effect of DEX is time dependent: removal of 100nM DEX after the day 0-2 treatment provided inhibition of sGAG loss only for the next 8 days. After this 8-day period, the absence of added DEX resulted in increased aggrecan catabolism, causing 1.8x higher cumulative GAG loss compared to the continuous DEX dose condition by day 16 (Fig 6.5).

6.3.5. Dose dependent bioactivity of Avidin-DEX compounds on bovine cartilage

As shown in Fig. 6.5, a low but continuous dose of 100nM DEX was sufficient in suppressing IL-1α induced GAG loss in bovine cartilage explants. The goal of this study is to develop a therapeutic approach to suppression of cytokine-induced GAG loss inside cartilage for a long enough period of time with only a SINGLE-dose treatment, which we hypothesize is possible using Avidin-conjugated DEX. Hence, it was necessary to determine the concentration of one dose of Avidin-delivered DEX that is effective in suppressing GAG loss for longer durations. For this, we tested the dose dependent biological response of PEG Avidin-ester-DEX (compound C) on IL-1 treated cartilage explants (Fig 6.6A). We found that one dose of all DEX concentrations (100nm-100μM) in compound C was effective in significantly suppressing IL-1-induced GAG loss; however, the effect was most prominent using the 100μM DEX-equivalent condition at 8 days (Fig 6.6A). Hence, a concentration of 100μM DEX delivered through Avidin conjugated DEX structures was chosen for subsequent experiments.

6.3.6. Single dose Avidin-DEX rescued GAG loss in the presence of IL-1α over 22 days

Fig. 6.6B compares the cumulative GAG loss over 22 days for 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). Treatment with IL-1α significantly increased GAG loss compared to untreated controls (Fig. 6.6B). When IL-1α treated explants were incubated with a single dose of soluble DEX from day 0-2, GAG loss was markedly reduced. However, treatment with a single dose of fast-releasing PEG Av-ester-DEX suppressed GAG loss even further than the soluble DEX alone. PEG Av-ester-DEX has a mean half-life of 14.4h (Fig 6.3B), suggesting that 70% of loaded DEX was released before the first medium change (i.e., at 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 at the early stages of this culture period.

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PEG Av-HZ-DEX inhibited sGAG loss in a manner similar to soluble DEX at earlier time points. In contrast to the effect of soluble DEX, which wore off by day 18, PEG Av-HZ-DEX showed more effective inhibition of sGAG loss by days 20 to 22. HZ is slow drug releasing linker with a mean half-life of 57.4h at pH4 (Fig 6.3C). Because it cleaves in acidic environments, HZ is expected to release DEX close to the negatively charged GAG chains near the chondrocyte surface (at which aggrecan concentration is highest), and also in the endosomal/lysosomal cell compartments, if endocytosed by cells [32]. Additionally, a decrease in pH from 7.4 to 5.5 has been reported at cartilage surface of OA patients. At the micro-scale, the densities of negatively charged aggrecans and chondrocytes increase with depth into cartilage from the superficial zone [33]. We previously showed that Avidin, due to its high positive charge, penetrated rapidly through the full thickness of bovine cartilage resulting in enhanced uptake (400x higher than its neutral counterpart) and binding with the negatively charged groups [23]. When conjugated, Avidin will potentially carry the drug into deep zones of cartilage where most chondrocytes reside. As a result, the rate of drug release from HZ linker is expected to increase with depth into cartilage. Finally, a 1:1 molar ratio of two linker chemistries with effective DEX concentration of 100µM performed better than the slow releasing HZ linker but similarly to ester linker (Fig. 6.6B) and is expected to yield greater rescuing effect at longer time points in vivo.

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

Compared to the untreated controls, sulfate incorporation was significantly suppressed by IL-1α treatment in bovine cartilage explants by day 8 of culture. Addition of a single dose of 100µM soluble DEX from day 0-2 or continuous dose of soluble 100nM DEX significantly rescued 35S-sulfate incorporation, which is accepted as a measure of sGAG biosynthesis (with >95% of sGAG associated with aggrecan in these young bovine explants [34]. PEG Av-ester-DEX and PEG Av-HZ-DEX also had similar rescuing effects. In addition, Avidin conjugated DEX structures also rescued chondrocyte viability in the presence of IL-1α (Figs. 6.7A-F). A dramatic increase in cell death was observed after treatment with IL-1 alone (1ng/mL) by day 8 (Fig. 6.7B). In contrast, addition of a single dose of 100µM DEX on day 0-2 (Fig. 6.7C) or a continuous dose of 100nM DEX (Fig. 6.7D) successfully prevented cell death induced by IL-1α. Treatment with a single dose of PEG Av-ester-DEX or PEG Av-HZ-DEX (Figs. 6.7E-F) added on day 0, also successfully rescued chondrocyte death similar to that observed using the soluble DEX conditions. Previously, we showed that Avidin itself did not affect chondrocyte biosynthesis of proteins and proteoglycans in similar-aged bovine cartilage explants in-vitro [15]. A high concentration of 100µM Avidin alone, however, showed 2x higher GAG loss compared to untreated control in ITS.
deficient medium, likely the result of reduced intra-tissue osmotic pressure that can cause decreased water content and release of some aggregcan [15]. In this work, we used one dose of < 25 μM Avidin for delivering 100μM DEX to cartilage explants in low glucose medium containing 1% ITS (often used, defined, serum-free culture medium with insulin replacing the insulin-like growth factor-1 (IGF-1) and other growth factors that suffer batch-to-batch variability in commercial serum). Therapeutic use of Avidin can elicit the production of anti-Avidin antibodies in-vivo [13,35,36] but this has been shown to have no effect on its safety or efficacy in humans [13,14,37]. Furthermore, PEGylation of Avidin has been shown to weaken and delay its immunogenic response in mice [16].

Avidin as a nanocarrier can transport candidate DMOADs (disease modifying OA drugs) into the deep zones of cartilage at a fast rate and bind reversibly thereby creating a drug reservoir and providing sustained delivery. High uptake of Avidin was demonstrated even after depletion of ~40% GAGs in cartilage [23], making it suitable especially for treating early stage PTOA wherein majority of the proteoglycan content is still intact. It is now well accepted that OA affects the entire joint and thus multiple DMOADs are needed for its effective treatment. One example of such drug combination is IGF-1, a pro-anabolic growth factor [38,39] known to stimulate cartilage repair and anti-catabolic glucocorticoid, dexamethasone [24]. IGF-1 can be similarly conjugated with Avidin to enable its targeted and sustained delivery in cartilage along with DEX.

### 6.4. Conclusion

Avidin is demonstrated as an effective nanocarrier to deliver and provide sustained release of DEX inside cartilage explants in an inflammatory OA-like model. Controlled release of DEX from Avidin was achieved by using a combination of ester (fast release) and hydrazone (slow release) linkers. Separately and together, these DEX delivering nanocarriers suppressed cytokine-induced catabolic effects over 3 weeks in an in-vitro organ culture system using only a single dose at the start of culture. Avidin is able to rapidly penetrate through the deep zones of cartilage and to remain bound within the tissue before being cleared from the synovial fluid in vivo, providing a novel method for delivering drugs to the tissue matrix, cells and other targets. Disease modifying as well as pain management therapeutics can be conjugated with Avidin using such simple chemical linkers. Our ongoing studies involve testing of Avidin drug conjugates in vivo using a well-accepted animal model of post-traumatic osteoarthritis (the rabbit anterior cruciate ligament transection (ACLT) model).
6.5. References


Figure 6.1 Schematic representation and chemical structures of the four Avidin (Av) conjugated dexamethasone (DEX) compounds formulated. (A) Av+DEX: Avidin supra-molecularly (non-covalent) loaded with DEX. (B) PEG Av+DEX: Pegylated Avidin supra-molecularly 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.
**Scheme 1:** Steps of synthesis for covalent conjugation of Avidin with PEGylated dexamethasone using ester linker

1. **Dexamethasone (1)**
   - Pyridine: reacts with **HO** to form **OH**
   - Reaction: 24h, rt.

2. **Dexamethasone succinate**
   - **NH2-PEG2.3K**
   - Reaction: EDCI, NHS, DMF

3. **Dexamethasone succinate**
   - **Amino PEG biotin**
   - Reaction: PBS, 7.4, 2.5 h

4. **Ester bond**
   - **Dexamethasone immobilized on Avidin**

**Summary:**
- **Steps:** Pyridine, EDCI, PBS
- **Reagents:** Pyridine, EDCI, NHS, DMF, PBS
- **Products:** Dexamethasone succinate, Amino PEG biotin, Ester bond, Dexamethasone immobilized on Avidin
Scheme 2: Steps of synthesis for covalent conjugation of Avidin with PEGylated dexamethasone using hydrazone linker

1. Dexamethasone (1)

2. Iminothiolane

3. Amino PEG biotin

4. PBS, 7.4

5. Acid-cleavable linker
Figure 6.2 (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.

Figure 6.3(A) In vitro DEX release profiles for non-covalently DEX loaded Avidin structures in PBS (pH 7.4) at 37°C.
Figure 6.3 (B) In vitro 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) DEX release profiles for PEG Av-hydrazone-DEX in PBS at 37°C at pH 7.4 (diamonds) and at pH 4 (squares). Hydrazone (HZ) 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.
Figure 6.4 Images of fluorescently stained bovine cartilage explants to check for chondrocyte viability after 48h incubation with (A) basal media, untreated control. Images of explants treated with Avidin conjugated DEX structures containing effective DEX concentration of 100μM with the following two linkers, (B) Ester and (C) Hydrazone. Green indicates viable cells and red indicates non-viable cells.

Figure 6.5 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 is presented as Mean +/-SEM, N=3 animals, n=6 explants for each treatment condition from each animal. All treatment conditions are significantly different from the untreated control. # represents significant difference from the continuous dose condition (p≤0.05)
Figure 6.6 (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 +/-SEM, N=1 animal, n=6-12 explants each treatment condition.

Figure 6.6 (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 (purple), compound D (blue) or 1:1 molar ratio of compounds C and D. 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. All conditions are significantly different from IL-1 alone (red). Soluble DEX (green) and compound D (blue) are significantly different from control (black) starting at day 4. Compound C (purple) and the combination C+D (yellow) are significantly different from control (black) starting at day 10 and day 8 respectively.
Figure 6.6 (C) 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 +/- SEM, N=4 animals for all conditions except for 1 dose of soluble DEX, where N= 3 animals, n=6-12 explants each condition from each animal. In Figs 6A and C, * represents significant difference compared to IL-1α alone condition, p<0.05.

Figure 6.7 Images of fluorescently stained bovine cartilage explants (4x objective) cultured for 8 days to check for chondrocyte viability in (A) basal medium, untreated control (B) treated with 1ng/ml of IL-1α alone. The following conditions were treated with IL-1α (1ng/ml) along with (C) 1 dose of 100μM soluble DEX (D) continuous dose of 100nM soluble DEX during 8 days of culture (E) 1 dose of PEG Av-ester-DEX and (F) 1 dose of PEG Av-HZ-DEX (final DEX concentration of 100μM in (E) and (F)). 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
Chapter 7  In-vivo efficacy of single dose Avidin-DEX treatment using rabbit ACL transection model

7.1. Design of Animal Study

This chapter discusses the design of an ongoing pre-clinical in-vivo test for safety and therapeutic efficacy of single dose Avidin-DEX compounds in a rabbit anterior cruciate ligament transection (ACLT) model of post traumatic osteoarthritis (PTOA). We saw in Chapter 5 that using larger animals than mice or rats is very important for enhancing transport-kinetics and intra-cartilage residence time of drugs and drug carrying particles in vivo. Thus, we used mature (12 month old) New Zealand female white rabbits, with average weight of 10 lbs. Generally by 8 months of age, the physes on growth plates in the distal femur and proximal tibia are closed, thereby defining animal maturity. In addition, the closed physes inhibit regeneration of rabbit articular cartilage once degradation sets in. This rabbit model is well-accepted for evaluating biological efficacy and mechanisms of therapeutic intervention which otherwise would be impossible to be studied in humans.

We discussed in previous chapters that traumatic injuries like ACL rupture or meniscus tears are followed by immediate escalated synovial fluid levels of metalloproteases and inflammatory cytokines, including IL-1, TNFα, IL-6, in human joints. It is critical to intervene at the time of injury to suppress these catabolic activities, and bring their levels down to a ‘normal’ state. The rabbit ACLT model, therefore, is an appropriate animal model to test the efficacy of the Avidin-DEX system for treatment of PTOA. The biological efficacy of single dose Avidin-DEX is compared to conditions using saline (vehicle) alone and commercially available soluble DEX.

7.2. Methods

The experiment design is shown in Fig. 7.1. In summary, the ACLs of the right knees of 60 rabbits were transected on day 0 and the contralateral left knees all underwent a sham surgery. One week post-surgery, the ACLT right knee was i.a. injected with 600 μl of the either (i) saline alone; (ii) soluble dexamethasone sodium phosphate (0.5mg); or (iii) Avidin-DEX (total DEX dose of 0.5mg). The contralateral left knees received saline injections. A 1:1 molar ratio of ester and hydrazone linker chemistry (Compound C, discussed in Chapter 6) was used in the Av-DEX formulation as it was shown
to provide the longest sustained release of DEX (Figure 6.6B). Table 7.1 shows a summary of treatments for the right and left knees of all 60 rabbits.

After i.a. injection, the rabbit knees were slowly flexed several times to distribute the injected materials uniformly throughout the joint space. Using a digital caliper, the medial-lateral joint width was measured before the surgery, on the day of i.a. injection, and then at the time of sacrifice to check for joint swelling. The rabbits were sacrificed either at 3 weeks or 9 weeks post-surgery to evaluate the short as well as long term rescuing effect of one-dose Avidin-DEX. We used N=10 rabbits for each treatment condition, thus total of 60 rabbits were used in this study.

After sacrifice, all joints were lavaged with PBS and synovial fluid samples were collected for mass spectrometry to analyze for protein levels of IL-1, IL-6, TNFα and aggrecan fragments. Gross morphology of joints was assessed qualitatively for osteophytes, soft tissue fibrillation & lesions. The lateral femoral condyle along with the trochlear groove was subjected to analysis using a high resolution computed tomography scanner. Data analysis such as quantification of osteophyte volume and thickness and density of subchondral bone are currently being conducted. The lateral femoral condyle was then later fixed and decalcified in Formal-4 (Decal Chemical Corporation) for 24-72 h for histology. OA severity is evaluated by histological scoring and selected immunohistochemical analyses. Articular cartilage from the medial tibial plateau and medial femoral condyle was collected and pooled as ‘Medial cartilage’. The Medial cartilage and the medial meniscus were flash frozen in liquid nitrogen to assess gene expression of IL-1β, ACAN, Col2, MMP-1, MMP-3, MMP-13, TNFα and ADAMTS-5 using quantitative real-time PCR (normalized by GAPDH).

The lateral tibial plateau cartilage (with intact tibia bone) and the lateral menisci were frozen in PBS containing proteinase inhibitors; on re-thawing, the biomechanical properties of the cartilage and menisci were measured using a Dynastat mechanical spectrometer. A 1mm diameter non-porous indenter fixed to the Dynastat’s load cell assembly was used to test three spots on the lateral tibial plateau and one spot on the lateral meniscus as shown in Fig. 7.2. At each spot on the tibial plateau surface, four consecutive displacements were applied to achieve steps in strain of 5%, 5%, 2.5% and 2.5%, for a total of 15% strain. After each step in strain, the transient stress relaxation response was continuously recorded. At 15% strain, sinusoidal waveforms were applied with frequencies ranging from 0.005 Hz to 2Hz; the dynamic modulus was thereby calculated as a function of frequency. Thereafter, these cartilage and meniscus samples were digested using Proteinase-K and then measured for total sGAG and collagen.
content using the DMMB assay and the hydroxyproline assay respectively. The details of how different
joints tissues were used for assaying are shown in Table 7.2.

7.2.1. DEX dose selection

In-vitro data from Chapter 6 suggests that a single dose of Av-DEX with effective DEX
concentration of 100μM is effective in suppressing cytokine induced GAG loss over 3 weeks. Assuming
that there is 2ml of synovial fluid in the inflamed rabbit joint and that about 33% of i.a. injected Avidin is
retained inside the joint after 24h (observation made from rabbit study presented in Chapter 5), a total of
0.25mg of DEX is needed to achieve DEX concentration of 100μM inside the rabbit joint after 24h of i.a.
injection. Glucocorticoid dose recommended for small animals like cats and dogs is 0.5mg per 5kg
weight. Thus we used a one-time dose of 0.5mg of DEX in our studies.

7.3. Results and Discussion

In this section, we discuss preliminary results as this analysis is still in progress. Fig 7.3 A-D
show mRNA levels of IL-1β, MMP-1, ACAN & MMP13 in medial cartilage from the three weeks study.
Gene expression of IL-1β, MMP-1 & ACAN was significantly suppressed with Av-DEX treatment
compared to operated control. In contrast, MMP13 gene expression increased significantly with DEX or
Av-DEX treatment compared to control.

Treatment with DEX or Av-DEX did not restore ACLT-induced loss of stiffness in lateral tibial
plateau cartilage (Fig 7.4 A) By 9 weeks, stiffness of the tibial plateau cartilage of ACLT knee was
restored and became greater than the control suggesting a recovery process. The dynamic stiffness also
decreased significantly after ACLT; values measured at 0.01Hz and 1HZ are shown in Fig. 7.4B-C. Thus,
even normal physiological loading conditions can affect articular cartilage more than usual. Additionally,
dynamic stiffness measured at 1Hz was ~2 times greater than that measured at 0.01Hz due to the
poroelastic self-stiffening behavior of cartilage (i.e., the dynamic stiffness of normal cartilage increases
with compression frequency). ACLT also resulted in greater GAG loss in the tibial plateau cartilage
compared to that in contralateral control knees at 3 weeks. The GAG levels, however, were restored to
normal by 9 weeks-saline treatment (Fig 7.5A), which also explains the restoration of equilibrium
stiffness of tibia cartilage observed in Fig. 7.4A. In patellar cartilage, collagen content remained
unchanged; however, GAG content reduced in Av-DEX treated patellar cartilage compared to control
(Fig 7.5 B-C).
The above described results provide suggestive evidence that a single Av-DEX treatment is more effective than the single soluble DEX condition in curbing injury-induced catabolic effects as observed through gene expression data. Av-DEX, however, resulted in higher GAG loss in patellar cartilage compared to DEX. This may be because of longer retention and exposure to either DEX or Avidin-DEX in the patellar cartilage owing to its greater thickness than other cartilage tissues. In addition, the i.a. injection was introduced through the patella tendon, which is in close proximity to patella cartilage. A more detailed experiment to understand and analyze biological activity of sustained retention of Avidin and DEX separately is now being conducted. These preliminary data also suggest that a pro-anabolic growth factor is necessary in addition to DEX to enable regeneration process for an effective treatment.
Figures and tables

Table 7.1. Summary of intra-articular treatments on knee joints of 60 rabbits

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<th>3 weeks</th>
<th>9 weeks</th>
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<td>Right knee</td>
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<td>Un-operated control</td>
<td>ACLT + Saline</td>
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<td>N=10 rabbits each time point</td>
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<td>Group 2</td>
<td>Sham surgery</td>
<td>ACLT + Soluble DEX</td>
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<td>N=10 rabbits each time point</td>
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<td></td>
</tr>
<tr>
<td>Group 3</td>
<td>Sham surgery</td>
<td>ACLT + Av-DEX</td>
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<tr>
<td>N=10 rabbits each time point</td>
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Table 7.2. Assays conducted on different joint tissues

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<td>Tibia plateau</td>
<td>Meniscus</td>
<td>Femoral condyle</td>
<td>Tibia plateau</td>
<td>Meniscus</td>
<td>Patella cartilage</td>
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<td>qPCR</td>
<td>X</td>
<td>X</td>
<td>X</td>
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<tr>
<td>GAG &amp; Collagen</td>
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<td>X</td>
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<td></td>
</tr>
</tbody>
</table>
N=10 each condition, Total =60 rabbits

0 1w 3 or 9w
ACLT i.a. treatment Tissue harvest

Figure 7.1 Design of rabbit study- the ACL of right knees of rabbits were transected on day 0. The left knee underwent a sham surgery. One week post-surgery, one of the following treatments was introduced intra-articularly in the right ACLT knee (i) saline, (ii) soluble DEX, and (iii) Av-DEX (compound C) with an effective DEX concentration of 100µM. N=10 rabbits were used for each treatment. The left knees received only saline. 3 weeks or 9 weeks post-surgery, rabbits were sacrificed and various joint tissues were harvested.

A. B.

Blue crosses: locations tested on lateral tibial plateau
Red crosses: location tested on lateral meniscus

Figure 7.2 (A) Image showing the experimental configuration for indentation tests using the Dynastat mechanical spectrometer to obtain biomechanical properties of tibial plateau cartilage. (B) Blue crosses represent the locations tested on the lateral tibial plateau cartilage where average stiffness values have been reported. Red cross represents the location tested on the lateral meniscus.
Figure 7.3 mRNA levels of (A) IL-1β (B) MMP1 (C) ACAN and (D) MMP13 in pooled articular cartilage from the medial femoral condyle and the medial tibial plateau. p values are based on Tukey’s post-hoc test for comparison between the multiple treatment conditions. We used p<0.05 for statistical significance. Values are expressed in Mean +/- SEM.
Figure 7.4 (A) Mean equilibrium modulus of the lateral tibial plateau cartilage. Dynamic stiffness measured in tibial cartilage at (B) 0.01 Hz and (C) 1 Hz. Values expressed in Mean+/-SEM. N=10 rabbits each treatment condition except Av-DEX, where N=9 rabbits.
Figure 7.5A  Rabbit Tibia Cartilage GAG

- Contralateral control (left knee)
- ACLT (right knee)

Figure 7.5B  Rabbit Patella Cartilage GAG

- Contralateral control (left knee)
- ACLT (right knee)

Figure 7.5C  Rabbit Patella Cartilage Collagen

- Contralateral control (left knee)
- ACLT (right knee)

Figure 7.5 sGAG concentration measured using DMMB dye assay in (A) lateral tibial plateau cartilage and (B) patella cartilage. (C) Collagen concentration measured using the hydroxyproline assay in the patella cartilage. Values expressed in Mean+/−SEM. N=10 rabbits each treatment condition except Av-DEX, where N=9 rabbits.
Chapter 8  Summary and Future Direction

8.1. Summary of Thesis

In this work, we first established that for drugs or drug carrying particles to penetrate through deep zones of articular cartilage, their hydrodynamic diameter has to be smaller than 10nm. We demonstrated that electrostatic interactions can be effectively used to enhance the diffusion-kinetics, uptake and binding of selected cationic nano-carriers inside the negatively charged connective tissues such as cartilage, meniscus and ligaments. We show that Avidin, a globular protein with 7nm diameter (MW 66kDa) and high positive charge (net charge between +6 and +20) exhibited ideal characteristics of a nano-carrier for intra-tissue drug delivery into cartilage. Avidin is able to penetrate rapidly into the deep zones of connective tissues and bind to the negatively charged aggrecans. Avidin resulted in 400x higher uptake than its neutral counterpart, NeutrAvidin, and penetrated through full thickness cartilage within 24h, while it took 4 days for NeutrAvidin to penetrate half the thickness. Moreover, Donnan partitioning of Avidin suggested six fold upward partitioning factor at the synovial fluid-tissue interface. This resulted in a steep intra-tissue concentration gradient that significantly enhanced its transport rate and uptake into cartilage. We found that the binding of Avidin with the negatively charged groups of cartilage is weak and reversible ($K_D=150 \; \text{M}$) and yet it has a long retention time owing to the high binding site density of the negatively charged groups ($N_T=2,920 \; \text{M}$).

Particles with diameter greater than 10nm can also be used for drug delivery into cartilage if they can be functionalized to bind with the tissue surface, as we showed by using 15 nm QDs. As these particles degrade, they can release the drug closer to the tissue targets than if they are freely floating in the synovial fluid. We proposed three mechanisms for particle based intra-tissue drug delivery in the order of their effectiveness in Chapter 3.

In Chapter 4, experiments showed that Avidin penetrated rapidly through different rat knee tissues resulting in a half-life of 29 hours inside articular cartilage, compared to NeutrAvidin which cleared out completely from the joint space within 24h. The shorter half-life, however, compared to the bovine in-vitro data of Chapter 2 is due to the thinner cartilage of rat compared to bovine. Rat cartilage is 10 times thinner than the bovine, and since transport kinetics within cartilage scale as square of tissue thickness, a 100 times faster rate of transport (and loss from the cartilage) is expected in the rat cartilage...
compared to bovine. Avidin showed a much longer half-life in the thicker rabbit cartilage; the longest measured half-life was 154 h in the medial tibial plateau cartilage which is also the thickest cartilage. Small animal models are suitable for understanding biological mechanisms, but it is critical to use larger animals for understanding transport phenomenon and drug delivery. While in small animals, the drug can diffuse in and out of the tissue rapidly, drugs might get cleared out from the joint space even before they begin penetrating into cartilage tissue in larger animals. We expedited the transport rate by using electrostatic interactions as demonstrated by Avidin. Once the drug is inside the cartilage, then it will take longer for it to diffuse out of a thicker cartilage than a thinner one. Thus we expect to see even better results in humans, as the cartilage is even thicker in adult human compared to adult bovine cartilage.

Next we used dexamethasone as an example drug for inhibiting OA-like cartilage degradation related to inflammatory catabolic effects. To do so, dexamethasone (DEX) was conjugated with Avidin. Glucocorticoids like DEX are small molecules that are rapidly cleared from the joint space, thus requiring multiple injections of high drug doses when used clinically via simple i.a. injections in humans and animals. We conjugated DEX to Avidin by using two chemical linkers, a fast releasing ester linker (mean half-life of 14 h) and a slow releasing pH cleavable hydrazone linker (mean half-life 57h at pH 4). We hypothesize that hydrazone linker will hydrolyze more in the tissue deep zones, which are rich in negatively charged aggrecan that make the microenvironment more acidic. A combination of these two linkers in our design enabled a sustained long term release of DEX, and is expected to provide both a fast release of drug to provide an immediate pain and inflammation relief to the patient as well as a sustained long term release of low doses of drugs in the next several days to inhibit progression to OA. We showed using an in-vitro cartilage explant culture model that Avidin-DEX was able to successfully suppress the IL-1 induced GAG loss over 3 weeks with only a single dose. A single dose of soluble DEX, however, had a shorter lived biological response and thus needed to be replenished continuously throughout the culture period. Our data suggest that a single intra-articular injection of Avidin-conjugated drug can enable sustained drug delivery in low doses and therefore has the potential to replace the current clinical practice of using multiple injections of high dose glucocorticoids in patients. Based on these results, Av-DEX formulation is currently being tested in a wellaccepted rabbit ACLT model of PTOA.

8.2. Future Work

Avidin-like structures can enable a unique means of intra-tissue local drug delivery into negatively charged tissues like cartilage. Other proteins that are smaller than 10nm diameter and exhibit similar properties like high positive charge and the ability to bind weakly and reversibly with cartilage
can be screened and studied for purposes of drug delivery. Some examples include lysozyme, monomers and dimers of Avidin, poly (arginine) chains etc. Synthetic peptides can also be designed and tested.

Avidin or Avidin-like structures can be conjugated with a variety of drugs such as growth factors, pain killers, and Fab-fragments of antibodies; applications to gene delivery are also feasible. One of the challenges for gene delivery into chondrocytes is to enable their penetration in the deep zone of cartilage where majority of the cells reside, and Avidin can enable this. We showed that Avidin can penetrate and remain bound inside other connective tissues such as meniscus and ligaments and thus be used for drug delivery and their repair. Avidin conjugated drugs can find their first applications for animal treatment and eventually target human trials. For example, the horseracing sector offers a major market opportunity and is much easier to enter due to the lower regulatory bars. Additionally, it will provide invaluable large-animal data that can further help in designing and staging human clinical trials. Other potential veterinary markets may include smaller animals like cats and dogs.

For further studies, it is recommended to synthesize these compounds in a GMP facility to minimize batch to batch variation. Additionally, chemical linkers other than ester and hydrazone can be screened to find the most suitable ones for the desired drug release profiles depending on the type of drug to be delivered. Finally, a more detailed animal study will help understand Avidin’s pharmacokinetics, pharmacodynamics and its immunogenic response (if there is any) in vivo.

Finally, the governing equations derived for modeling kinetics inside the joint space can be developed into an extensive computational model which might have applications in initial screening of drugs/drug carriers based on their residence time in the joint space.