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Avidin as a model for charge driven transport into cartilage and drug delivery for treating early stage post-traumatic osteoarthritis

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ABSTRACT

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 \sim 150$ μM) of Avidin to intratissue sites in cartilage. The large effective binding site density ($N_T \sim 2920$ μM) within cartilage matrix facilitates Avidin's retention, making its structure suitable for particle based drug delivery into cartilage.

1. Introduction

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. 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]), 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

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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]. 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]. 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].

It is also essential 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 [16,17]. 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 [18–22] but the effects of particle size and surface morphology on their penetration, binding and retention within cartilage are less well understood [23]. The relative utility of 15 nm micelles vs. 138 nm liposomes was recently reported, showing the need to further differentiate between size and structure [24].

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 [25], while the distance between GAG chains on aggrecan is only 2–4 nm apart from each other [26]. Aggrecan density increases with depth into cartilage from the surface (superficial) zone and restricts the ability of solutes to penetrate and diffuse within cartilage [27]. Maroudas et al. [27,28] 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 [29]) 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 [27], while the positively charged growth factor IGF-1 (pI 8.5) partitions upward into cartilage [30]. 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 [31]; 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. 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.

2.1. 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 [32]. Briefly, cylindrical cartilage disks (3 mm or 6 mm diameter) were cored using a dermal punch and then sliced to obtain the top 1 mm 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 Ca^{2+}/Mg^{2+}) supplemented with protease inhibitors (Complete Protease Cocktail tablet in 50 mL PBS, Roche Applied Science, IN) in a 37 °C, 5% CO_2 incubator for 24–48 h.

2.2. Solutes types

2.2.1. 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 [33]).

2.2.2. 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 (8 kDa) 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 1.

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. 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. 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.