Green fluorescent proteins engineered for cartilage-targeted drug delivery: Insights for transport into highly charged avascular tissues


A R T I C L E   I N F O

Keywords:
Post-traumatic osteoarthritis
Cartilage
Drug delivery
Engineered supercharged green fluorescent proteins (GFP)
Cationic drug carriers
Charge

A B S T R A C T

Osteoarthritis (OA), the most common form of arthritis, is a multi-factorial disease that primarily affects cartilage as well as other joint tissues such as subchondral bone. The lack of effective drug delivery, due to the avascular nature of cartilage and the rapid clearance of intra-articularly delivered drugs via the synovium, remains a major challenge in the development of disease modifying drugs for OA. Cationic delivery carriers can significantly enhance the uptake, penetration and retention of drugs in cartilage by interacting with negatively charged matrix proteoglycans. In this study, we used “supercharged” green fluorescent proteins (GFPs), engineered to have a wide range of net positive charge and surface charge distributions, to characterize the effects of carrier charge on transport into cartilage in isolation of other factors such as carrier size and shape. We quantified the uptake, extent of cartilage penetration and cellular uptake of the GFP variants into living human knee cartilage and bovine cartilage explants. Based on these results, we identified optimal net charges of GFP carriers for potential drug targets located within cartilage extracellular matrix as well as the resident live chondrocytes. These cationic GFPs did not have adverse effects on cartilage in terms of measured cell viability and metabolism, cartilage cell biosynthesis and matrix degradation at doses needed for drug delivery. In addition to quantifying the kinetics of GFP uptake, we developed a predictive mathematical model for transport of the GFP variants that exhibited the highest uptake and penetration into cartilage. This model was further used to predict the transport behavior of GFPs during scale-up to in vivo applications such as intra-articular injection into human knees. The insights gained from this study set the stage for development of cartilage-targeted delivery systems to prevent cartilage degeneration, improve tissue regeneration and reduce inflammation that may cause degradation of other joint tissues affected by OA.

1. Introduction

Osteoarthritis (OA) is a multi-factorial disease with multiple causes and risk factors that leads to degeneration of cartilage and associated joint tissues in ~90 million adults in the United States [1]. There are currently no disease modifying drugs (DMOADs) that slow or reverse the progression of OA [2]. A major challenge to development of DMOADs is that symptoms usually appear late in disease progression when the joint has already been extensively or irreversibly damaged [3]. However, increasing attention is being paid to post traumatic OA (PTOA), caused by the combination of acute mechanical impact and the inflammatory cascade that follows traumatic joint injuries (e.g., rupture of the anterior cruciate ligament). PTOA develops in ~50% of all patients who suffer a traumatic joint injury, especially women [4], and accounts for 12% of all OA cases [5]. Since the initiating event that triggers PTOA is known, clinicians can potentially intervene early to...
prevent the progression of the disease if methods for efficacious DMOAD delivery were available. Indeed, recent clinical trials focusing on treatment after joint injuries have been initiated based on this hypothesis [6,7].

While there are promising DMOAD candidates for PTOA, none have been proven to be effective in slowing or reversing the progression of disease [2,8]. It is now accepted that lack of appropriate delivery to cartilage has been a major impediment to drug discovery [9,10]. Since cartilage is avascular, direct intra-articular (IA) injection of drugs into the joint synovial fluid can minimize potential systemic side effects [9]. However, IA therapy currently remains ineffective due to poor cartilage penetration of drugs and rapid clearance of drugs from the joint via the synovial capillaries and lymphatics [9].

We recently discovered that the cationic nanoparticle Avidin, a 66 kDa positively charged protein, can act as a carrier that dramatically improves both penetration and retention in cartilage, exemplified by delivery of the glucocorticoid, dexamethasone, to cartilage. While these studies demonstrated that cationic carriers can significantly improve delivery to cartilage, the precise effect of carrier charge density on penetration and transport into cartilage remains unknown.

Proteins can be engineered to carry a wide range of net theoretical charge while maintaining almost identical mass, structure and function. This is achieved by substituting solvent-exposed surface amino acids, which do not play a crucial role in protein folding, with either positively charged or negatively charged amino acids [14]. For example, supercharged green fluorescent proteins (S-GFPs) are engineered by mutating a robust GFP variant called superfolder GFP. Cationic S-GFPs have been reported with net positive charges from +6 to +48 [14,15]. Additional variants of GFP have been engineered to have approximately zero net charge, but with focal regions of positive and negative surface charge which sum to zero [16]. Proteins engineered in this manner can be used to gain fundamental insights into the role of the net charge as well as the spatial distribution of particle surface charge on transport and delivery into tissues, in isolation of other factors such as molecular weight or protein shape. Engineered GFPs (MW ~28 kDa; hydrodynamic diameter ~5 nm) are significantly smaller than the upper size limit for penetration into cartilage (~15 nm [10]), providing a potential advantage for delivering large biologic drugs in addition to small molecule therapeutics. Additionally, while certain cartilage-targeted carriers (like Avidin) have been shown to be unable to enter cartilage cells [17], supercharged GFPs can be endocytosed into mammalian cells with high efficiency [14]. Thus, these GFPs may be effective carriers for drug delivery to both intracellular and extracellular matrix targets.

In this study, engineered GFPs were used to quantify the effect of net charge and the distribution of surface charge on transport into and through full thickness human and bovine cartilage, independent of other factors such as size and shape that can also affect transport. Specifically, uptake and penetration into cartilage tissue explants was quantified and visualized for four S-GFP variants having net theoretical charges of +9, +15, +25 and +36. In addition, three neutral GFP variants with different surface charge distributions were used as controls for the effects of net charge, and to characterize the effects of local surface charge distribution on transport and binding/retention inside cartilage. The uptake of the four S-GFP variants into chondrocytes within and outside native cartilage explants was also visualized. The results of these extracellular and intracellular uptake studies suggest optimal choices of S-GFP(s) for delivering to intracellular versus extracellular targets. Dose response experiments were carried out to identify any potential adverse or toxic effects of S-GFPs on chondrocyte viability, metabolism, cellular biosynthesis, as well as adverse effects on cartilage extracellular matrix. Finally, dynamic uptake experiments were performed to measure the reaction rate constants for GFP binding in cartilage; the results were used to develop a predictive mathematical model for the transport of GFPs both in vitro and in vivo systems.

2. Materials and methods

2.1. Structure and preparation of cationic and neutral GFP variants

The structures and the electrostatic surface potentials of the four cationic supercharged GFP and three neutral GFP variants are shown in Fig. 2A and B respectively. The surface charge patch distributions of the Janus and HP neutral GFPs are shown in Figs. S1 and S2.

Cationic S-GFPs: BL21 Star (DE3)-competent E. coli cells were transformed with plasmids encoding the supercharged GFPs with a His6 N-terminal purification tag. A single colony was grown overnight in Luria-Bertani (LB) broth containing 50 mg/mL chloramphenicol at 37 °C. The cells were diluted 1:100 into 1 L of the same media and grown at 37 °C until OD600 0.6–0.7. The cultures were incubated on ice for 60 min before induction of protein expression with 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) (GoldBio). Expression was sustained for 15 h with shaking at 16 °C. Cells were collected by centrifugation at 6,000g for 20 min (4 °C) and resuspended in cell collection buffer (100 mM tris(hydroxymethyl)-aminomethane (Tris)-HCl, pH 8.0, 1 M NaCl, 20% glycerol, 5 mM tris(2-carboxyethyl)phosphine (TCEP; GoldBio), 0.4 mM phenylmethane sulfonyl fluoride (Sigma-Aldrich) and 1 complete, EDTA-free protease inhibitor pellet (Roche) per 50 mL buffer used). Cells were lysed by sonication (5 min total, 3 s on, 5 s off) and the lysate cleared by centrifugation at 22,500g (25 min). The cleared lysate was incubated with His-Pur nickel nitrilotriacetic acid (nickel-NTA) resin (1.5 mL resin per litre of culture, Thermo Fisher) with rotation at 4 °C for 60 min. The resin was washed with 20 column volumes of cell collection buffer before bound protein was eluted with elution buffer (110 mM tris(hydroxymethyl)-aminomethane (Tris)-HCl, pH 8.0, 0.5 M NaCl, 20% glycerol, 5 mM TCEP (GoldBio), 200 mM imidazole). The resulting protein fraction was further purified on a
5 mL Hi-Trap HP SP (GE Healthcare) cation exchange column using an Akta Pure FPLC. An NaCl gradient (from 0 to 1 M) was used to elute the supercharged GFP from the HP SP column; the NaCl concentration required to elute the S-GFP increased with an increase in protein charge.

Protein-containing fractions were concentrated using a column with a 10 kDa cutoff (Millipore) centrifuged at 3,000 g, and the concentrated solution was sterile-filtered through a 22-mm polyvinylidene difluoride (PVDF) membrane (Millipore). After concentration, proteins were resuspended in storage buffer (100 mM tris(hydroxymethyl)-aminomethane (Tris)-HCl, pH 8.0, 0.5 M NaCl, 20% glycerol, 5 mM TCEP (GoldBio)). After sterile filtration, proteins were quantified using a NanoDrop instrument, sterile-filtered through a 22-mm PVDF membrane and stored at −80 °C. The protein sequences for all the GFP variants are listed in section S1 in the Supplementary section.

**2.2. Bovine cartilage explant and chondrocyte harvest**

Cartilage tissue was harvested from the femoropatellar grooves (Fig. 3A) of 11 knee joints from 11 different freshly slaughtered 1–2 week old bovine calves (Research 87, Boylston MA) as previously described [19]. Cylindrical cartilage disks (3 mm in diameter) that included the intact superficial zone were cut out and washed with Dulbecco’s phosphate buffered saline without calcium and magnesium (PBS). They were then trimmed down to 1 mm thick explants and equilibrated for 48 h at 37 °C and 5% CO2 in serum-free medium (Low glucose (1 g/L) Dulbecco’s Modified Eagle’s medium (DMEM), 100 U/mL penicillin, 100 mg/mL streptomycin, 0.25 mg/mL amphotericin, 10 mM HEPES buffer, 0.1 mM nonessential amino acids (NEAA), 0.4 mM proline and 20 mg/mL ascorbic acid).

To extract live chondrocytes, cartilage tissue slices were first harvested from the femoral condyles of bovine joints. Solutions of collagenase (1.25 mg/mL) and pronase (2 mg/mL) were prepared in low glucose DMEM with 5% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, 0.25 mg/mL amphotericin, 10 mM HEPES buffer, 0.1 mM nonessential amino acids (NEAA) and 0.4 mM proline. After washing with PBS, cartilage slices were first incubated in the pronase solution for 1 h followed by overnight incubation in the collagenase solution at 37 °C and 5% CO2. The final solution was then
passed through a 70 μm filter and a 40 μm filter to remove any undigested cartilage fragments. The filtrate was centrifuged at 400 g for 8 min and the pellet was resuspended in 50 mL PBS. Viable cell counts were obtained from aliquots of this suspension using a hemocytometer and Trypan blue. The cells were then centrifuged out of the suspension (400 g, 8 min), resuspended in freezing medium (FBS with 5% DMSO) and stored in liquid nitrogen until use.

### 2.3. Human cartilage explant harvest

Two human knee joint distal femurs were obtained through the Gift of Hope Organ and Tissue Donor Network (Itaska, IL). A Collins grade 0 normal knee was from a 36 year male donor and a near-normal grade 1 from a 35 year female. (The Collins visual grading scale grades spans grades 0–4 [20], with grade 0 = normal and grade 4 = end stage osteoarthritis.) All procedures were approved by the Rush University Medical Center Institutional Review Board (ORA Number: 08082803-IRB01-AM01) and the Committee on the Use of Humans as Experimental Subjects at MIT. Femoral cartilage plugs (3 mm diameter, Fig. 3B) were harvested using a biopsy punch, trimmed down to 1 mm thickness and equilibrated for 48 h (37 °C, 5% CO₂) in high glucose (4.5 g/L) DMEM which was supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, 0.25 mg/mL amphotericin, 10 mM HEPES buffer, 0.1 mM nonessential amino acids (NEAA), 0.4 mM proline and 20 mg/mL ascorbic acid.

### 2.4. Bovine synovial fluid

Bovine synovial fluid (BSF) was procured from Lampire Biological Laboratories (Pipersville, PA). BSF was collected using sterile syringes from the carpal joints of adult cows within 1 h of slaughter, pooled and delivered at 10 °C within two days of collection. Some aliquots of BSF were kept frozen at −20 °C (for short-term storage) and the rest were stored at −80 °C.

### 2.5. Quantitative uptake of GFPs into cartilage

Experiments to quantify the uptake of GFPs into cartilage were started after the explants had been pre-equilibrated in culture medium for 48 h post-harvest. Sterile PBS solutions containing 1% bovine serum albumin (BSA) along with 1 μM concentration of each of the four S-GFP variants (+36, +25, +15 and +9) and the three neutral GFP variants (Janus, NP and HP) were prepared. The BSA was included in the PBS to minimize loss of GFPs in the solution caused by non-specific binding to the plasticware. Since the main function of the albumin in these experiments was to prevent such non-specific binding in the external solution, independent of tissue species, we used BSA for the bovine cartilage as well as the human cartilage experiments (as opposed to BSA with bovine cartilage and human serum albumin (HSA) with human cartilage) to maintain consistency in experimental techniques. Groups of 5 cartilage explants (matched for position along the joint surface) were washed with PBS, and each explant was separately incubated in 250 μL of the GFP solutions in sterile polypropylene vials for 24 h or for 8 days (37 °C, 5% CO₂). (Standard 96-well cell culture plates were not used because S-GFPs could stick to polystyrene even in the presence of BSA, leading to a loss of protein. In contrast, use of polypropylene with 1% BSA enabled complete recovery of fluorescence.) In addition, uptake of the cationic S-GFPs into cartilage explants was also performed in solutions of bovine synovial fluid. On the day of the experiment, BSF was thawed and warmed in a 37 °C water bath with periodic, gentle agitation. At the end of the incubation period in either PBS or BSF, cartilage explants were washed with PBS and transferred to a solution of 10X PBS + 1% BSA for desorption. At the same time, the fluorescence of the absorption bath was measured using a plate reader (Synergy H1, Biotek Instruments Inc.) (excitation: 485 nm, emission: 526 nm). This was used along with a standard curve to obtain the concentration of GFP left behind in the absorption bath. At the end of the desorption in 10X PBS + 1% BSA, cartilage explants were weighed and the fluorescence of the desorption bath was measured. This fluorescence measurement was used with a standard curve and the explant wet weights to calculate the concentration of GFP inside cartilage explants (mol/mg wet weight). The uptake ratio was calculated as the ratio of GFP concentration inside cartilage (mol/mg wet weight) to the final absorption bath concentration (mol/mL).

### 2.6. GFP penetration into cartilage and chondrocytes

Bovine cartilage explants were incubated for either 24 h or 8 days...
(37 °C, 5% CO₂) in 1 μM solutions of the cationic S-GFPs dissolved in PBS + 1% BSA. Similarly, explants were placed in 1 μM solutions of the neutral GFPs for either 18 h or 26 h. In a separate series of experiments, bovine cartilage explants were cultured in ultra-low attachment cell culture plates (Corning) for a period of 8 days in 1 μM solutions of cationic S-GFPs dissolved in low glucose (1 g/L) DMEM (with no phenol red) with 10% fetal bovine serum (FBS) supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, 0.25 mg/mL amphotericin, 10 mM HEPES buffer, 0.1 mM nonessential amino acids (NEAA) and 0.4 mM proline (37 °C, 5% CO₂). Media changes were performed every 48 h, and fresh cationic S-GFP were added with each media change. At the end of all such experiments, explants were sectioned longitudinally into ~100 m slices and washed with PBS. Primary bovine chondrocytes were incubated for 24 h (37 °C, 5% CO₂) in 100 nM solutions of GFPs dissolved in serum-free medium having the same composition as that described in the bovine cartilage harvest section. At the end of 24 h, chondrocytes were washed three times with heparin dissolved in PBS (20 U/mL) to remove non-internalized GFPs, fixed with 4% paraformaldehyde, stained with DAPI and washed with PBS.

Explant slices and fixed chondrocytes were imaged at 10 × magnification using a confocal microscope (Olympus Fluoview FV1000), and Z-stacks were obtained with a voxel depth of 4.22 μm for cartilage and 1.16 μm for chondrocytes. 3D cartilage Z-stacks were flattened to 2D using the Z project function available in the Fiji software package. During imaging, some cartilage explants were too large to fit into the field of view of the confocal microscope. In these cases, two overlapping Z-stacks were captured and combined using the Stitching plugin available in Fiji [21] before performing 2D Z projections. For chondrocyte Z-stacks, the slice in which the cell images were the sharpest (best focus) were used.

2.7. Effects of cationic GFPs on GAG loss, cell viability and metabolism in cartilage explants

10 nM, 100 nM and 1 μM solutions of each of the cationic S-GFP variants were prepared in low glucose (1 g/L) DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 0.25 mg/mL amphotericin, 10 mM HEPES buffer, 0.1 mM NEAA, 0.4 mM proline and 20 mM ascobic acid. Groups of 5 bovine cartilage explants were incubated in these solutions in ultra low attachment cell culture plates (Corning) at 37 °C, 5% CO₂. Untreated controls consisted of groups of 5 explants incubated in medium without any GFPs. Medium changes were carried out every 48 h for the 8-day duration of the experiment. Treatment groups received fresh GFP doses with each medium change. Explant viability by day 8 was measured using the Alamar Blue assay (ThermoFisher). Cartilage explants were incubated for 2.5 h in a 1X solution of Alamar Blue dissolved in culture medium. Absorbance measurements at 570 nm and 600 nm were performed using a microplate reader (Synergy H1 Hybrid Multi-Mode Microplate Reader, BioTek) and used to calculate the percentage reduction of Alamar Blue, which is directly proportional to the explant viability. These values were normalized by explant weights. These weight-normalized readings for the treatment groups were then normalized by the weight-normalized mean value for the untreated controls. To measure chondrocyte biosynthesis rates, explants were cultured in the presence of 5 μCi/mL 35S-sulfate radiolabel between days 6 and 8 of culture, and the rate of incorporation of radioactive sulfate into sulfated glycosaminoglycans (sGAGs) was measured [19]. At the end of the experiment, all explants were washed with PBS with non-radioactive sulfate to wash out excess radiolabel, weighed and digested with proteinase K. Liquid scintillation counter (PerkinElmer) was used to measure the amount of radiolabel incorporated within the explant extracellular matrix. Radiolabel incorporation was normalized by the duration of exposure to radiolabel (48 h) and by the DNA content of the explants (measured using the Hoechst 33258 dye binding assay [22]).

To calculate the cumulative loss of GAGs from the explant that may have been caused by the 8-day culture with various cationic S-GFPs, the amount of sGAG lost to the medium and that left behind in the explants at the end of the experiment was measured using the dimethylene blue (DMMB) dye binding assay [23].

2.8. Dynamic uptake: transport experiments and theory

To measure the kinetics of the uptake of cationic S-GFPs into cartilage, dynamic uptake experiments were performed. Cartilage disks (6 mm diameter) were clamped to one end of a transport chamber in such a way that one face was in contact with an impermeable wall and the other face had a circular area with a 4 mm diameter exposed to a well-mixed bath (see Results). The initial bath was a 1X PBS + 1% BSA solution (total volume = 2 mL). At time zero, + 9 S-GFP was added to the bath, and the bath concentration was monitored continuously through fluorescence measurements. A mathematical diffusion-reaction model was formulated (Supplementary section S4) and used to predict the changes in bath concentration of + 9 S-GFP upon transport into the cartilage explant disks. Diffusion-reaction parameters obtained from the fit were then used in the model to predict the numerical values of the final uptake ratios obtained experimentally at 24 h and 8 days.

2.9. Statistical analysis

Statistical analysis was carried out using MATLAB R2015a software. All quantitative data are plotted as Mean ± 95% Confidence Interval. Mean values and standard deviations are also reported in tables in the supplementary material. Depending on the experiment, one-way or two way ANOVAs were also performed followed by the post-hoc Tukey’s HSD test; p-values less than 0.05 were considered statistically significant.

3. Results

All cationic supercharged GFPs used in this study were very similar in size and also had high purity as shown by the gel in Fig. S3. We therefore used these GFPs to investigate the effect of cationic charge on transport into cartilage tissue independent of any other factors that may affect transport properties. Neutral GFPs (as characterized in Fig. S4 and Table S1) with varying surface charge distributions were used as controls for net charge and to further test whether the surface charge distribution and charge patch size of net-neutral GFPs might affect cartilage penetration.

3.1. Effect of charge on the uptake and transport of cationic carriers into cartilage

Cationic S-GFPs were dissolved in either PBS + 1% BSA or bovine synovial fluid (BSF) and their uptake ratios were quantified in bovine cartilage tissue after 24 h and 8 day incubations (Fig. 4A–D and Table S2). All the cationic S-GFP variants showed enhanced uptake into cartilage (Fig. 4) and in every condition tested, the lower charged variants had a significantly higher uptake ratio compared to the higher charged variants. Additionally, it was observed that the uptake of the cationic S-GFPs from PBS + 1% BSA was higher than their uptake from BSF (Fig. 4, (A) vs (C), (B) vs (D)). Finally, the uptake ratios of S-GFPs at the 8 day time point were higher than their uptake ratios at 24 h (Fig. 4, (A) vs (B), (C) vs (D): note differences in y axis scaling).

The higher uptake ratios of cationic S-GFPs at 8 days compared to 24 h indicated that transport into the cartilage explants had not reached equilibrium in the first 24 h, i.e., that the GFPs had not penetrated through the full thickness of the cartilage explants within 24 h. To visualize the extent of penetration, bovine cartilage explants that were incubated for 24 h in culture medium with 1 μM concentrations of the cationic S-GFPs were sliced longitudinally and imaged using confocal...
microscopy (Fig. 4E). +9 S-GFP penetrated the full 1 mm thickness of cartilage in 24 h while the extent of penetration decreased as the cationic charge of the GFP variant increased. Similar experiments were performed with continuous doses of cationic S-GFPs that were replenished every 48 h over an 8 day period and the extent of tissue penetration was visualized every 24 h from Day 1 to Day 8 (see Fig. S5).

3.2. Effect of surface charge distribution on uptake of net neutral GFPs into cartilage

Uptake ratios of the three neutral GFPs into bovine cartilage after a 24 h incubation in solutions of the GFPs in 1X PBS + 1% BSA (37 °C and 5% CO2) are shown in Fig. 5 and Table S3. Two of the three neutral variants had an uptake ratio of ~1, i.e., the concentration inside the cartilage is the same as that in the bath, and there is no enhanced uptake. However, at the same 24 h time point, Janus GFP showed an enhanced uptake ratio of 10.1 ± 1.3 (Fig. 5A, Table S3). The 24 h uptake ratios of each of the cationic S-GFPs from PBS + 1% BSA were at approximately the same level or higher than that of the Janus GFPs, with the highest uptake ratio at approximately 25 for the +9 S-GFP at the same conditions.

Following the 24 h incubation in GFP solutions, cartilage explants were transferred to either [1X PBS + 1% BSA] or [10X PBS + 1% BSA] solutions for an additional 48 h, and the percentage of GFP inside the
Explants that were incubated for 24 h or 8 days in solutions of either PBS + 1% BSA or in BSF (Fig. 6C,D and Table S4). The results were consistent with those obtained using bovine tissue in that the uptake ratios increased as the cationic S-GFP charge decreased and the 8 day uptake ratios were much higher than the 24 h uptake ratios. Tissue from the 36 year old male donor was used to obtain the 24 h uptake ratios of cationic S-GFPs dissolved either in PBS + 1% BSA or in BSF (Fig. 6C,D and Table S4). Similar to bovine cartilage, the uptake from PBS + 1% BSA was higher than the uptake from BSF, and the uptake ratios of +36 and +25 GFPs were significantly lower compared to the other two GFP variants. However, unlike bovine cartilage, +15 GFP (and not +9) had the highest uptake ratio. The difference in the uptake ratios of +15 and +9 GFPs was statistically significant in the case of uptake from BSF (Fig. 6D), but not in the case of PBS + 1% BSA (Fig. 6C).

3.4. Chondrocyte uptake of cationic S-GFPs increases with net charge

Primary bovine chondrocytes were harvested and incubated in solutions of 100 nM cationic S-GFPs dissolved in culture medium. Control cells were incubated in medium with no GFPs. Confocal microscopy (Fig. 7), performed with fixed settings for fluorescence imaging, revealed that the cellular uptake of cationic S-GFPs into chondrocytes increased significantly with increasing net charge. These results are consistent with trends observed in other cell types such as HeLa cells [24].

In a separate experiment, the cellular uptake of the three neutral GFP variants was tested using the same method (24 h incubation in 100 nM solutions followed by fixation and confocal microscopy with fixed settings). In this experiment, a negative control with no GFPs and a positive control with +9 GFP was included. The results shown in Fig. 8 demonstrate that the cellular uptake of the neutral GFPs is much lower compared to that of +9 GFP.

We also tested the hypothesis that higher cationic charged GFPs could be taken up by chondrocytes within native cartilage tissue. To visualize the extent of intracellular uptake of S-GFPs, bovine cartilage explants that were incubated for 24 h or 8 days in solutions of either +36 or +9 GFP in PBS + 1% BSA were sliced longitudinally and imaged using confocal microscopy (Figs. 9 and 10). The +9 GFP penetrated the full 1 mm thickness of cartilage in 24 h while +36 GFP did not (Fig. 9). However, by day 8, +36 GFPs were found to be localized primarily inside chondrocytes throughout the tissue thickness while the +9 GFPs were found in the extracellular matrix (ECM) of the explant, but not within the cells (Fig. 10). Thus, the trend of cell uptake versus net cationic charge found within intact cartilage explants was similar to that found with isolated cells (Fig. 7).

3.5. Effect of cationic S-GFPs on cartilage matrix sGAG loss, chondrocyte biosynthesis rates and cell viability in native cartilage tissue

Bovine cartilage explants were treated with 1 μM, 100 nM and 10 nM doses of each of the four cationic S-GFP variants used in this study. An untreated control group was also included. 10 nM and 100 nM doses of cationic S-GFPs did not lead to any changes in the DNA content (Fig. 11A), cell viability (Fig. 11B), aggrecan biosynthesis as a measure of cell metabolic activity (Fig. 11C), or sGAG release from explant (Fig. 11D). At 1 μM doses, there was a statistically significant but modest increase in the sGAG loss (Fig. 11D) from explants and a statistically significant decrease in their biosynthesis rate (Fig. 11C). But these doses did not lead to significant changes in DNA content (Fig. 11A) or cell viability (Fig. 11B). The mean and standard deviations for all the outcome measures are reported in Table S5.

Dynamic uptake of +9 GFP into both human and bovine cartilage (the former from the 35 year old female donor knee) was measured using the experimental configuration of Fig. 12A. As the GFP entered the cartilage explants, the bath concentration was measured continuously using real-time fluorescence detection, and observed to decrease with time over a 48 h period (see Fig. 12B, Figs. S7 and S9). A mathematical model that included the effects of diffusion and charge-based partitioning and binding was developed for the transport of GFPs from the bath into cartilage tissue (equations 17 to 22, Supplementary section S4). The diffusivity of +9 GFP (Table S6) was estimated based on the empirical power law model formulated for cartilage transport [25], accounting for solute hydrodynamic radius and steric interactions within cartilage extracellular matrix, but not charge. The positive charge of the GFP will lead to a high Donnan partitioning coefficient at the bath/cartilage interface, as seen previously for penetration of Avidin into cartilage [10] as well as previously reported Donnan equilibrium measurements of ions in cartilage [26] and low molecular...
weight peptide drugs [27]. GFP charge can also lead to binding interactions with cartilage matrix which were incorporated through a reaction rate term in the model. Since the bath volume and cartilage dimensions were known along with estimates for diffusivity, the only remaining unknown parameters in the model were the partition coefficient and the reaction rate constant for binding of +9 S-GFP to matrix sites. These parameters were calculated by fitting the model to the experimental data (Table S6). The partition coefficient was found to be 10.9 for human cartilage and 11.2 for bovine cartilage, while the reaction rate constants were $1.2 \times 10^{-7}$ and $6.4 \times 10^{-8}$, respectively. The resulting model fit, as well as the sensitivity of model predictions to values of partition coefficient and reaction rate are shown in Figs. S7 and S8 for human cartilage and Figs. S9 and S10 for bovine cartilage. The model fit for the dynamic uptake experiment using human cartilage is also shown in Fig. 12B.

The mathematical equations for transport (both bath and tissue) are identically applicable to the dynamic uptake experiments and the transient uptake experiments with measurements at 24 h and 8 days shown above in Figs. 3, 4 and 6. (The only differences between the two experiments were the bath volume and the size of the cartilage disks used.) The model was thereby used to predict the bath concentration and uptake ratio when the bath volume and cartilage dimensions were changed to those of the uptake experiments in Fig. 3. Using values of the diffusivity, partition coefficient and reaction rate constant identical to those for the dynamic uptake experiment, the predicted values of 24-h and 8-day uptake of +9 GFP compare favorably with the experimental data (Tables S7 and S8).

Finally, the model was used to predict the transport of +9 GFPs into human knee cartilage for the case of intra-articular injection into the knee joint. The diffusivity, partition coefficient, and reaction rate constant were assumed to be the same as the values for the dynamic uptake experiment while literature values were used for human knee synovial fluid volume and cartilage thickness [28–31]. A full numerical solution for +9 GFP concentration in synovial fluid and cartilage was computed as a function of time using MATLAB. In a separate simulation, the differential equation for the synovial fluid bath was modified to account for continuous clearance of synovial fluid components by the joint capsule synovium tissue. The results for the synovial fluid concentration and the average concentration inside cartilage as a function of time for the case study of a human knee are shown in Figs. S13 and S14. The sensitivity of these predictions to changes in the partition coefficient and the reaction rate constant are shown in Figs. S15 to S18.

4. Discussion

The inability to deliver different classes of drugs to cartilage in a sustained manner remains a major challenge in developing disease modifying drugs for OA and PTOA. The use of positively charged delivery carriers [10] that can specifically target cartilage through electrostatic interactions with the highly negatively charged cartilage extracellular matrix (Fig. 1) is a promising new approach to overcome this challenge. In this study, we demonstrated that while positive charge...
improves nanocarrier uptake and penetration into cartilage, higher charge does not necessarily lead to better penetration and transport through the full thickness of tissue, and that there are optimal ranges of charge for effective delivery. We used engineered cationic supercharged GFPs (S-GFPs) carrying net charge ranging from $+9$ to $+36$ but having similar sizes and shapes (Fig. 2A). Three additional engineered GFPs with neutral net charge (Fig. 2B) served as controls and also to study the effects of surface charge distribution on penetration and transport. S-GFP variants having net charge of $+9$ and $+15$ had significantly higher uptake ratios compared to those for higher charged S-GFPs ($+25$ and $+36$) or neutral GFPs. These trends were observed with both human (Fig. 6) and bovine cartilage (Fig. 4). Additionally, $+9$ and $+15$
S-GFPs exhibited a faster transport rate in cartilage and were able to penetrate the full thickness of the tissue at a much earlier time point compared to the +25 and +36 variants (Figs. 4E and 9 and Fig. S5). Quantitatively, this corresponded to much higher uptake ratios after cartilage incubation with GFPs for 8 days (Fig. 4(B,D) and 6B) compared to 24 h incubations (Fig. 4(A,C) and 6(A,C)). As the net charge of a delivery carrier increases, upward Donnan partitioning at the bath-cartilage interface increases, which would accelerate transport into the tissue [32]. However, carriers with higher cationic charge can also exhibit stronger binding interactions with the densely packed

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Fig. 8. Cellular uptake of neutral GFP variants in primary bovine chondrocytes is significantly lower than that of cationic S-GFPs. Cells were incubated in medium with no GFP (controls), Janus GFP, NP GFP, HP GFP and +9 GFP. Brightfield images (left column) are shown alongside fluorescence images for DAPI staining (blue, middle column) and GFPs (green, right column). Increasing brightness in green fluorescence indicates higher intracellular GFP concentration since the microscope and laser settings were the same for all fluorescence images. Scale bar: 60µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
negatively charged sulfated glycosaminoglycans (sGAGs) of aggrecan proteoglycans in cartilage. A higher coulombic attraction occurs with increasing cationic charge, thus yielding a strong electrostatic driving force for complexation with sGAGs. In addition to leading to a favorable decrease in enthalpy, electrostatic binding of cationic GFP molecules with the negatively charged aggrecan chains can also lead to the release of large numbers of counterions (such as Na\(^+\)) that were previously tightly bound through condensation onto the aggrecan chains. This counterion release leads to a favorable increase in entropy \([33]\), which acts as a strong driving force that stabilizes the GFPs in the bound state to the aggrecans. Additionally, while both arginine and lysine residues have similar contributions to the net charge of cationic S-GFPs, the guanidino group of arginine is known to exhibit much stronger binding with sulfated GAG \([34]\) compared to lysine. The number of arginines in

Fig. 9. Confocal microscopy images showing the 24 h penetration of cationic and neutral GFPs from PBS + 1% BSA into bovine cartilage explants. The first column shows the fluorescence images for GFPs while the second column shows the location of the cells inside the tissue (nuclei have been stained with Hoechst 33258 dye). The third column is the overlay of the first two columns, and shows the distribution of the GFPs with respect to the cells. Scale bar: 200\(\mu\)m.
cationic S-GFPs increases monotonically with increasing charge (from 11 in +9 GFP to 20 in +36 GFP). While stronger binding leads to greater retention of the GFPs in cartilage (with the potential for sustained drug release), such binding could slow transport of the GFPs into the tissue, consistent with the decreased uptake and penetration observed with increasing charge (Figs. 4 and 6).

Importantly, we also found that the uptake from synovial fluid (BSF) (Fig. 4(C,D) and 6D) was 20–50% lower than that from PBS + 1% BSA (Fig. 4(A,B) and 6C) for all S-GFPs by 24 h. Synovial fluid is a dialysate of plasma [35] that contains proteins such as albumin and the negatively charged glycosaminoglycan, hyaluronan (HA), secreted by joint capsule synovium. These synovial fluid constituents can bind S-GFPs and thereby slow their transport into cartilage. As such, the effective dose of GFP-bound drug would have to be titrated accordingly for clinical translation, since molecules injected into the synovial fluid have a ∼1–24 h half-life [36] in the joint before being cleared by the joint capsule.

While net cationic charge dramatically affected transport into

Fig. 10. Cellular uptake of S-GFPs in bovine chondrocytes inside native tissue increases with cationic charge. Bovine cartilage explants were incubated in solutions of GFPs dissolved in PBS + 1% BSA for 8 days. They were then sectioned (same orientation as Fig. 3E) and imaged using confocal microscopy. The first column shows the fluorescence images for GFPs while the second column shows the location of the cells inside the tissue (nuclei have been stained with Hoechst 33258 dye). The third column is the overlay of the first two columns, and shows the distribution of the GFPs with respect to the cells. The first two rows show that +9 GFP is localized mostly in the cartilage extracellular matrix, while +36 GFP is predominantly located in the cells. Scale bar: 200μm.
Fig. 11. 8-day continuous dose response results for bovine cartilage explants treated with 10 nM to 1 μM doses of S-GFPs. An untreated control group was also included. (A) S-GFPs do not lead to any change in the cellularity (as measured by the DNA content) of cartilage explants. (B) Cartilage explant viability, as measured by the Alamar Blue assay on day 8 of the experiment, remains unaffected by different doses of S-GFP. (C) S-GFPs cause a statistically significant decrease in the chondrocyte biosynthesis rate as measured by the normalized incorporation rate of radioactive 35S-sulfate during days 6–8 of the experiment. (D) S-GFPs cause a slight but statistically significant increase in the cumulative percentage sulfated glycosaminoglycan (sGAG) loss at a dose level of 1 μM but not at lower doses. 9–10 cartilage explants (that were matched for joint region) from 2 animals were used for each condition and the individual data points, mean and 95% confidence interval are reported; *: p < 0.05; **: p < 0.01; ***: p < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 12. Dynamic transport experiments for quantifying transport properties of delivery carriers in cartilage (A) Experimental set-up for dynamic uptake transport experiments: Human or bovine cartilage is clamped to one side of the transport chamber with one side facing an impermeable wall and the opposite face exposed to a well mixed solution of + 9 GFP in PBS + 1%BSA. The concentration of + 9 GFP in the bath is measured continuously through fluorescence measurements (see Supplementary Fig. S6). (B) Dynamic uptake transport data (solid blue line) and model fit (dashed black line) for + 9 GFP transport in human cartilage. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
cartilage, we also demonstrated that surface charge distribution of net neutral carriers can significantly affect their transport properties. The net-neutral NP and HP GFPs had an uptake ratio of ~1 (Fig. 5A and Table S3), consistent with the absence of Donnan partitioning or electrostatic binding interactions. However, the net-neutral Janus GFPs had a ten-fold higher uptake within 24 h than the NP and HP GFPs. While the NP and HP GFPs have multiple small patches of both positive and negative charge (Fig. 2B), the Janus GFP has one large positive and one large negative patch, each encompassing approximately half its surface (Fig. 2B and Fig. S1). The positive patch of Janus GFP may enable it to interact more effectively with negatively charged cartilage GAGs, complexing with negative segments and locally displacing counterions (e.g., Na+), thus driving directional transport into the tissue. While the precise mechanism relating surface charge distribution to transport of net-neutral carriers into cartilage warrants further investigation, this discovery broadens the choice of potential carriers for drug delivery into such avascular tissues.

In addition to high uptake of a drug delivery carrier, an understanding of the kinetics of penetration and uptake is critically important since drugs would need to enter cartilage before clearance by joint capsule capillaries and lymphatics within a few hours. We formulated a mathematical model to predict the time scales for penetration into cartilage based on tissue dimensions, bath volume and carrier properties (i.e., diffusivity, interfacial partition coefficient and the rate constant for binding with cartilage matrix components). Based on the experimental configuration for dynamic uptake (see Fig. 12A and Supplementary Section S4), we derived expressions for carrier bath concentration as a function of time and their relation to the temporal evolution of the carrier concentration profile inside cartilage. The partition coefficient and the binding reaction rate constant for +9 GF were estimated by fitting model predictions to data from dynamic uptake experiments. The value of the partition coefficient was found to be approximately the same in both bovine and human tissue (Table S6). However, the binding reaction rate constant and was ~5-fold higher for bovine cartilage compared to human cartilage (Table S6). This reaction rate constant is directly proportional to the binding site density (Supplemental Equation 13) which we believe is associated with the concentration of cartilage aggrecan-GAGs. Aggrecan density in the superficial zone of cartilage can vary by a factor of 2–4 over a single joint surface depending on the location along the joint and the animal/human from whom it was harvested. Aggrecan density increases with depth in cartilage, and the superficial zone is thinner in young bovine cartilage compared to adult human cartilage. Therefore, we would expect the aggrecan density to be higher and more uniform in its distribution near the surface in bovine cartilage, which would result in a higher density of binding sites. Additionally, aggrecan density is highest at birth, and we would expect cartilage from newborn bovine calves to have higher aggrecan density compared to adult human cartilage. Therefore the difference in the reaction rate constants falls within the expected variability based on differences in aggrecan concentration.

The model was able to give good predictions of the measured bath concentration in the uptake experiments (shown schematically in Fig. 3) at the 1- and 8-day time points (Table S7). It was then used to predict the transport behavior of +9 GFs injected into the human joint, both in the presence and absence of clearance by the joint capsule. Specifically, predictions were generated for the concentration of GFs inside cartilage as a function of depth and time, and for the bath (synovial fluid) concentration as a function of time. The results for the bath (synovial fluid) concentration and the thickness-averaged cartilage concentration as a function of time obtained from these computer simulations are shown in Figs. S13 and S14. Based on these model results, the transport through human knee cartilage is predicted to be approximately an order of magnitude faster than the rate of uptake in the experiments of Figs. 4 and 6. The faster transport rate is primarily due to the much higher cartilage surface area to bath volume ratio inside the human knee compared to the in vitro uptake experiments. In the in vitro experiments (results shown in Figs. 4 and 6), we found that ~50% of +9 GF could penetrate human and bovine cartilage in 24 h (Table S7). Model predictions suggest that in human joints, an equivalent fractional change in the joint space concentration of +9 GF would take place in less than 2 h. Therefore, we do not expect the time required to enter and penetrate into human joint cartilage will be a hurdle when translating this work to clinical applications. Ongoing work includes extending this model to the other GFP variants (both cationic and neutral) to characterize their transport properties in cartilage.

Taken together, the +9 GF exemplifies the optimal carrier for delivering drugs that have targets either in the extracellular matrix or cell surface receptors of cartilage. Potential disease modifying therapeutics for post-traumatic osteoarthritis include anti-catabolic and anti-inflammatory glucocorticoids which act intracellularly, as well as pro-anabolic biologics which act at cell surface receptors. Trends for direct cellular uptake were greatly improved with higher cationic charge (e.g., Figs. 7 and 10), which is opposite to that for extracellular uptake.

Ongoing studies now focus on conjugation of anti-catabolic and pro-anabolic drugs to these GFP carriers, and quantifying delivery, first using explant organ co-cultures. Since there has been potential concern about the effects of cationic nanocarrier charge on cell viability [37,38], we first studied the effects of increasing S-GFP dose and increasing S-GFP charge on (a) proteolysis of matrix aggrecan that would lead to release of GAGs (one of the earliest events in PTOA), (b) chondrocyte matrix biosynthesis within living native explants, (c) ex-plant viability and (d) DNA content (Fig. 11). Cartilage explants were treated continuously for 8 days with cationic S-GFP doses ranging from 10 nM to 1 μM. S-GFP doses equal to or less than 100 nM had no adverse effects on any of these critical parameters. Even though the bath concentrations were 1 μM when adverse effects started appearing, the concentration inside the tissue would have been at least an order of magnitude higher in these in vitro studies due to enhanced uptake. In a previous study [19], a continuous dosage with a combination of 100 nM dexamethasone and 15 nM insulin-like growth factor 1 (IGF-1) was sufficient to protect human and bovine cartilage viability, degradation of matrix and loss of cell biosynthesis caused by inflammatory mediators that are upregulated immediately after traumatic joint injury. This previous study was performed without a drug carrier at conditions where the concentration of drugs inside cartilage would be approximately the same as the bath concentration. Due to the high uptake in cartilage, 10–100 nM doses of S-GFPs conjugated to similar drugs would be sufficient to maintain the requisite drug concentrations inside cartilage and achieve the same efficacy. The required carrier concentrations are therefore at least an order of magnitude lower than the threshold of 1 μM at which GFPs can lead to adverse effects.

5. Conclusions

Recent studies have demonstrated the conceptual advantage of using cationic nanocarriers such as Avidin to achieve enhanced, sustained delivery of low molecular weight drugs (i.e., dexamethasone) to cartilage, both in in vitro [17] and in vivo systems [39]. Use of such cationic carriers takes advantage of enhanced interfacial partitioning, accelerated transport and increased retention within cartilage due to electrostatic interactions with the tissue’s high density of negatively charged aggrecan proteoglycans [10]. The present study has focused on the important need to identify optimal nanocarrier net charge for delivery to both extracellular and intracellular targets in cartilage and other nearby joint tissues affected by traumatic joint injury. To achieve these aims, we quantified the effect of net charge of carriers on uptake and penetration into cartilage using green fluorescent proteins (GFPs) engineered to carry a wide range of net charge and while retaining nearly identical sizes and shapes. In addition, by using three net-neutral GFPs having different surface charge distributions, we demonstrated that carrier uptake and penetration into cartilage also depends
dramatically on the size and distribution of surface charge patches. These engineered GFPs have the advantage of being small enough to deliver both small molecule drugs and large biologics to cartilage. Optimal charge ranges are identified for uptake directly into cartilage cells, which makes these carriers highly effective for drugs with intracellular targets. Results have revealed optimal net charge of GFPs for the delivery efficacy to intracellular as well as ECM/cell surface receptors. For the dose ranges of interest for delivery, we demonstrated that cationic GFPs do not have adverse effects on cartilage matrix turnover, cell viability and metabolic rate. Finally, we developed a mathematical model to guide choice of delivery carriers and to estimate the rate constants for the binding of GFPs in cartilage that would provide sustained retention for delivery.

Author contributions

YK designed the study, conducted the experiments, analyzed and interpreted the data, formulated the mathematical model and fit to experimental data, and prepared the manuscript. HR and SK expressed and purified S-GFPs and neutral GFPs respectively, and assisted in the preparation of this manuscript. CR assisted with harvesting bovine cartilage tissue and with setting up dose response experiments. BH and DL contributed to the design of experiments and revised the manuscript. AJG contributed to the design of the study, interpretation of experimental data, formulation of the mathematical model, preparation of the manuscript and critical revision of the manuscript. All authors have approved the final version of this manuscript.

Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

Acknowledgements

This work was funded by DOD CDMRP grant W81XWH-14-1-0544, NSF MRSEC grant DMR-14-19807 and NIH/NCATS grant UG3 TR002186. The production of the Janus, homogeneous patch, and non-patch GFP variants was supported by the DOE Basic Energy Sciences under grant DE-SC0007106. H.A.R. and D.R.L. acknowledge US NIH press grants 1ROI DE-SC0007106. H.A.R. and D.R.L. acknowledge US NIH Post-doctoral fellowship 1F32 AR069884-01A1. CR assisted with harvesting bovine cartilage and with setting up dose response experiments. PH, BO and DL contributed to the design of experiments and revised the manuscript. AJG contributed to the design of the study, interpretation of experimental data, formulation of the mathematical model, preparation of the manuscript and critical revision of the manuscript. All authors have approved the final version of this manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.biomaterials.2018.08.050.

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[10] H.A.R. and D.R.L. acknowledge US NIH Post-doctoral fellowship 1F32 AR069884-01A1. CR assisted with harvesting bovine cartilage and with setting up dose response experiments. BH and DL contributed to the design of experiments and revised the manuscript. AJG contributed to the design of the study, interpretation of experimental data, formulation of the mathematical model, preparation of the manuscript and critical revision of the manuscript. All authors have approved the final version of this manuscript.

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References


