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Cover Letter

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To the Editors of the Journal of Visualized Experiments:

In response to the invitation from Sr. Science Editor Benjamin Werth, we hereby submit an original methods-based manuscript titled, "Design of Experimental Methods for the Characterization of Intra-Cartilage Transport Properties of Cationic Peptide Carriers."

The experimental protocols detailed in this work describe procedures used for characterizing the transport of cationic peptide carriers (CPCs) through cartilage tissue, where drug delivery remains an outstanding challenge. Since tissues like cartilage possess a high negative charge as a result of densely packed aggrecans, positively charged carriers can be used to enhance the transport of drugs, creating a depot within the tissue. For an optimal therapeutic response, drugs must penetrate deep within the tissue to reach their matrix and cellular target sites; thus, studying the transport of drugs and their carriers is critical towards predicting biological response. The experimental methods used for targeting cartilage, allow for quantification of the equilibrium uptake, depth of penetration and non-equilibrium diffusion rate of CPCs. The results obtained from these experiments provide a measure of solute concentration in cartilage, a visual representation of solute penetration through cartilage and an assessment of the strength of binding interactions between the cartilage matrix and drug carriers. Together, the results obtained from these transport experiments provide a guideline for designing optimally charged drug carriers which take advantage of weak and reversible charge interactions for drug delivery to negatively charged tissues.

The experimental designs can also be applied to characterize transport of drugs and drug-drug carrier conjugates in cartilage, which is beneficial for assessing therapeutic success prior to biological studies.

These methods can be adapted for the assessment of charged based delivery to several other negatively charged tissues in the body such as meniscus, cornea, vitreous humor and intervertebral disc to treat numerous degenerative diseases such as osteoarthritis, macular degeneration and low back pain.

Potential reviewers who have relevant experience include Christopher Price from the University of Delaware (cprice@udel.edu), Lawrence Bonassar from Cornell University (lb244@cornell.edu) and Behdad Pouran from MILabs (b.pouran@umcutrecht.nl).

Thank you for your consideration, and we look forward to hearing back from you.

Sincerely,
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TITLE:

Characterization of Intra-Cartilage Transport Properties of Cationic Peptide Carriers

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KEYWORDS:
electrostatic interactions, cationic peptide carriers, negatively charged tissues, electro-diffusive transport, fixed charge density, targeted drug delivery

SUMMARY:
This protocol determines equilibrium uptake, depth of penetration and non-equilibrium diffusion rate for cationic peptide carriers in cartilage. Characterization of transport properties is critical for ensuring an effective biological response. These methods can be applied for designing an optimally charged drug carriers for targeting negatively charged tissues.

ABSTRACT:
Several negatively charged tissues in the body, like cartilage, present a barrier to the targeted drug delivery due to their high density of negatively charged aggrecans and, therefore, require improved targeting methods to increase their therapeutic response. Because cartilage has a high negative fixed charge density, drugs can be modified with positively charged drug carriers to take advantage of electrostatic interactions, allowing for enhanced intra-cartilage drug transport. Studying the transport of drug carriers is, therefore, crucial towards predicting the efficacy of drugs in inducing a biological response. We show the design of three experiments which can quantify the equilibrium uptake, depth of penetration and non-equilibrium diffusion rate of cationic peptide carriers in cartilage explants. Equilibrium uptake experiments provide a measure of the solute concentration within the cartilage compared to its surrounding bath, which is useful for predicting the potential of a drug carrier in enhancing therapeutic concentration of drugs in cartilage. Depth of penetration studies using confocal microscopy allow for the visual representation of 1D solute diffusion from the superficial to deep zone of cartilage, which is important for assessing whether solutes reach their matrix and cellular target sites. Non-equilibrium diffusion rate studies using a custom-designed transport chamber enables the measurement of the strength of binding interactions with the tissue matrix by characterizing the

diffusion rates of fluorescently labeled solutes across the tissue; this is beneficial for designing carriers of optimal binding strength with cartilage. Together, the results obtained from the three transport experiments provide a guideline for designing optimally charged drug carriers which take advantage of weak and reversible charge interactions for drug delivery applications. These experimental methods can also be applied to evaluate the transport of drugs and drug-drug carrier conjugates. Further, these methods can be adapted for the use in targeting other negatively charged tissues such as meniscus, cornea and the vitreous humor.

INTRODUCTION:

Drug-delivery to negatively charged tissues in the body remains a challenge due to the inability of drugs to penetrate deep into the tissue to reach cell and matrix target sites[1]. Several of these tissues comprise of densely-packed, negatively-charged aggrecans which create a high negative fixed charge density (FCD)[2] within the tissue and act as a barrier for the delivery of most macromolecules[3,4]. However, with the assistance of positively charged drug carriers, this negatively charged tissue barrier can actually be converted into a drug depot via electrostatic charge interactions for sustained drug delivery[1,5] **(Figure 1)**.

[Place **Figure 1** here]

Recently, short-length cationic peptide carriers (CPCs) were designed with the goal of creating small cationic domains capable of carrying larger sized therapeutics for delivery to the negatively charged cartilage[4]. For effective drug delivery to the cartilage for treating prevalent[6,7] and degenerative diseases such as osteoarthritis (OA)[8], it is critical that therapeutic concentrations of drugs penetrate deep within the tissue, where a majority of the cartilage cells (chondrocytes) lie[9]. Although there are several potential disease modifying drugs available, none have gained FDA approval because these are unable to effectively target the cartilage[10,11]. Therefore, evaluation of the transport properties of drug carriers is necessary for predicting the effectiveness of drugs in inducing a therapeutic response. Here, we have designed three separate experiments that can be utilized for assessing the equilibrium uptake, depth of penetration and non-equilibrium diffusion rate of CPCs[4].

To ensure that there is a sufficient drug concentration within the cartilage that can provide an optimal therapeutic response, uptake experiments were designed to quantify equilibrium CPC concentration in cartilage[4]. In this design, following an equilibrium between the cartilage and its surrounding bath, the total amount of solute inside the cartilage (either bound to the matrix or free) can be determined using an uptake ratio. This ratio is calculated by normalizing the concentration of solutes inside the cartilage to that of the equilibrium bath. In principle, neutral solutes, whose diffusion through the cartilage is not assisted by charge interactions, would have an uptake ratio of less than 1. Conversely, cationic solutes, whose transport is enhanced via electrostatic interactions, show an uptake ratio greater than 1. However, as shown with CPCs, use of an optimal positive charge can result in much higher uptake ratios (greater than 300)[4].

Although high drug concentration within the cartilage is important for achieving therapeutic benefit, it is also critical that drugs diffuse through the full thickness of the cartilage. Therefore,

studies showing the depth of penetration are required to ensure that drugs reach deep within the cartilage so that the matrix and cellular target sites can be reached, thereby providing a more effective therapy. This experiment was designed to assess the one-way diffusion of solutes through cartilage, simulating diffusion of drugs into cartilage following intra-articular injection in vivo. Fluorescence imaging using confocal microscopy allows for the evaluation of depth of penetration into cartilage. Net particle charge plays a key role in moderating how deep drugs can diffuse through the matrix. An optimal net charge based on a tissue FCD is required to allow for weak-reversible binding interactions between cationic particles and the anionic tissue matrix. This implies that any interaction is weak enough so that particles can disassociate from the matrix but reversible in nature so that it can bind to another matrix binding site deeper within the tissue[4]. Conversely, excessive positive net charge of a particle can be detrimental towards diffusion, as too strong matrix binding prevents detachment of particles from the initial binding site in the superficial zone of cartilage. This would result in an insufficient biological response as a majority of the target sites lie deep within the tissue[9].

To further quantify the strength of the binding interactions, analysis of drug diffusion rates through cartilage is advantageous. Non-equilibrium diffusion studies allow for the comparison of real-time diffusion rates between different solutes. As drugs diffuse through the superficial, middle and deep zones of cartilage, the presence of binding interactions can greatly alter diffusion rates. When binding interactions are present between drugs and the cartilage matrix, it is defined as the effective diffusivity (D_{EFF}). In this case, once all binding sites have been occupied, the diffusion rate of drugs is governed by the steady-state diffusion (D_{SS}). Comparison between the D_{EFF} of different solute determines the relative binding strength of solutes with the matrix. For a given solute, if the D_{EFF} and D_{SS} are within the same order of magnitude, it implies that there is minimal binding present between the drug and matrix during diffusion. However, if D_{EFF} is greater than D_{SS}, substantial binding of particles to matrix exists.

The designed experiments individually allow for the characterization of solute transport through the cartilage, however, a holistic analysis inclusive of all results is required for designing an optimally charged drug carrier. The weak and reversible nature of charge interactions controls particle diffusion rate and allows for high equilibrium uptake and rapid full depth penetration through cartilage. Through equilibrium uptake experiments, we should look for carriers that show high uptake as a result of charge interactions which can be verified using non-equilibrium diffusion rate studies. However, these binding interactions should be weak and reversible in nature to allow for full-thickness penetration of the solute through cartilage. An ideal drug carrier would possess an optimal charge which enables strong enough binding for uptake and high intra-cartilage drug concentrations, but not too strong as to impede full-thickness diffusion[4]. The presented experiments will assist in the design characteristics for charge-based tissue targeting drug carriers. These protocols were used for characterizing CPC transport through cartilage[4], however, these can also be applied to a variety of drugs and drug carriers through cartilage and other negatively charged tissues.

PROTOCOL:

University approvals were obtained for conducting the experiments with dead tissues. Bovine

joints were obtained commercially from a slaughterhouse.

1. Cartilage explant extraction

1.1 Using a scalpel (#10 blade), cut and remove fat, muscles, ligaments, tendons and all other connective tissue to expose the cartilage from the femoropatellar groove of bovine knee joints.

1.2 Using 3 mm and 6 mm dermal punches, make perpendicular punches into the cartilage to extract cylindrical plugs. Immediately place the plugs in individual wells of a 48-well plate containing 500 µL of 1x phosphate buffered saline (PBS) supplemented with 1% v/v antibiotic-antimycotic.

1.3 Place the superficial side of a cartilage plug facing down into a well in the slicing fixture **(Figure 2)**. Using a razor blade, slice the plug along the surface of the slicing fixture to obtain a 1 mm thick cartilage explant that is inclusive of the superficial zone. Repeat for each cartilage plug.

1.4 Store cartilage explants individually in polypropylene tubes containing 500 µL of 1x PBS supplemented with protease inhibitors (PBS-PI, 1 PI mini-tablet per 50 mL 1x PBS) at -20 °C.

1.5 Prior to conducting each of the following transport experiments, thaw the explant-containing vials for 30 min in a 37 °C water bath.

[Place **Figure 2** here]

2. Equilibrium uptake of CPCs in cartilage

2.1 Gently dab cartilage explants (3 mm dia. X 1 mm thick.) with a delicate task wipe to remove excess 1x PBS from the explant surface. Using a balance, quickly record the wet weight of each explant and then immediately place in a 1x PBS bath to prevent dehydration.

2.2 Prepare 30 µM solutions (300 µL per explant) of fluorescently labeled CPCs in 1x PBS-PI. Use RNase-free polypropylene tubes for reconstitution.

2.3 In a 96-well plate, pipette 300 µL of each 30 µM CPC solution into separate wells. Avoid using wells near the edge of the plate to prevent evaporation. Using a spatula, transfer each explant to the solution containing wells.

2.4 Fill surrounding wells with 300 µL of 1x PBS and cover the well plate with lid. Seal the edges of the plate with flexible film to minimize evaporation.

2.5 Inside of a 37 °C incubator, place the plate on a plate shaker to limit the particle sedimentation. Incubate for 24 h under gentle rotation (50 rpm with a 15 mm orbit) to allow for the equilibrium uptake of CPCs in the cartilage **(Figure 3)**.

2.6 Generate a standard curve for correlation of fluorescence to CPC concentration

2.6.1 Prepare serial dilutions of CPC solutions from 30 µM – 0 µM (10 2-fold dilutions) in 1x PBS-PI in polypropylene tubes. Ensure that at least 500 µL of each dilution is present.

2.6.2 Add 200 µL of each dilution to consecutive wells in a black 96-well plate. Duplicate in another row to increase sample size.

2.6.3 Obtain fluorescence readings of each sample using a plate reader at the excitation and emission wavelengths of the fluorescent label using a plate reader.

2.6.4 Plot fluorescence reading vs. CPC concentration and derive an equation for the linear portion of the curve.

NOTE: To limit the variability in fluorescence readings, incubate the CPC stock solution under the same conditions as the sample plate prior to generation of the standard curve.

2.7 After 24 h of incubation, collect the equilibrium bath from each well in separate polypropylene tubes.

2.8 Transfer 200 µL of each solution into separate wells of a black 96-well plate. Obtain fluorescence readings of each sample under the same fluorescent settings as for the standard curve. If necessary, dilute the sample in 1x PBS-PI to ensure readings fall within the linear portion of the standard curve.

[Place **Figure 3** here]

3. Depth of penetration of CPCs in the cartilage

3.1 Prepare 30 µM solutions (300 µL per explant) of fluorescently labeled CPCs in 1x PBS-PI. Use RNase-free polypropylene tubes for reconstitution.

3.2 Using a scalpel, cut cartilage explants (6 mm diameter x 1 mm thickness) in half to make half-disks. Keep the explant hydrated with a layer of 1x PBS-PI while cutting.

3.3 Glue a half-disk explant into the middle of one well of the custom-designed 1-dimensional transport chamber using an epoxy **(Figure 4, Figure 5)**. Ensure epoxy is applied to the circumferential (curved) side of the explant. Remove excess glue from the well to prevent contact with the diffusion surface area of cartilage and make a note of the superficial side of the explant.

3.4 Add 80 µL of 1x PBS-PI to both sides of the explant. Pipette the liquid up and down from one side of the explant to check for leakage to the other side. If leakage occurs, readjust explant and apply epoxy as needed.

3.5 Replace the 1x PBS-PI from the side facing the superficial surface of cartilage (upstream) with 80 µL of 30 µM CPC solution. Maintain 80 µL of 1x PBS-PI on the side facing the deep zone of cartilage (downstream).

3.6 Carefully place the transport chamber in a cell culture dish. Cover the base of the dish with a layer 1x PBS to avoid evaporation of solutions. Ensure that there is no direct contact between solutions from upstream and downstream chambers.

3.7 Place the cell culture dish on a plate shaker to limit particle sedimentation. Incubate for either 4 or 24 h at room temperature under gentle rotation (50 rpm with a 15 mm orbit).

3.8 After incubation, remove the explant from chamber and cut ~100 µm slice from the center of the explant.

NOTE: This cross-section is inclusive of the superficial, middle and deep zones of cartilage.

3.9 Place the slice between a glass slide and a coverslip. Hydrate the slice with a layer of 1x PBS-PI.

3.10 At 10x magnification, image through the full thickness of the slice to obtain z-stack of fluorescent images using a confocal microscope.

3.11 Using ImageJ project the average intensity of the images within the z-stack to determine the depth of penetration of CPCs in cartilage.

3.11.1 Open the image stack by clicking on **File | Open**.

3.11.2 Click on '**Image**' on the task bar and click **Image | Stacks | Z Project** from the dropdown menu.

3.11.3 Input slice numbers from 1 to the final slice. Select '**Average Intensity**' under **Projection Type**. Click '**OK**.'

[Place **Figures 4** and **Figure 5** here]

4. Non-equilibrium diffusion rate of CPCs in the cartilage

4.1 Bring the two halves of the custom-designed transport chamber **(Figure 6)** together to assemble and close the chamber. Use washers, nuts and bolts to tightly close the chamber with a wrench.

NOTE: The transport chamber must be translucent as to not interfere with fluorescent readings. The transport chambers used in this protocol are made from polymethylmethacrylate (PMMA).

4.2 Coat the inner space of the chamber with 0.5% w/v non-fat bovine milk solution in 1x PBS (2 mL for each chamber) for 15 min to prevent non-specific binding of CPCs to chamber walls. Then rinse the chamber with 1x PBS (2 mL for each chamber).

4.3 Using the custom-designed slicing fixture **(Figure 2)** and a razor blade, slice a 6 mm diameter cartilage explant (transverse plane) to a thickness of 500-800 µm, inclusive of the superficial zone. Keep the explant hydrated with 1x PBS.

4.4 Using hammer-driven and dermal punches, create gaskets from rubber sheets as shown in **Figure 7**.

4.5 Assemble each half transport chamber to include 1 large rubber gasket, 1 PMMA insert and 1 small rubber gasket each. Place the explant in the wells of the plastic insert, with the superficial zone facing the upstream chamber. Sandwich the two halves together to complete the assembly and screw tightly using a wrench **(Figure 7)**.

4.6 Fill the upstream chamber with 2 mL of 1x PBS-PI and observe the downstream chamber for leakage of fluid from the upstream chamber. If leakage is present, reassemble the chamber, adjusting gasket position and tightness of screws. If no leakage, fill the downstream chamber with 2 mL 1x PBS-PI as well.

4.7 Add a mini-stir bar to both up and downstream chambers and place the chamber on a stir plate. Align the chamber so that laser from the spectrophotometer is focused towards the center of the downstream chamber. Place the signal receiver portion of the spectrophotometer behind the downstream chamber **(Figure 8)**.

NOTE: The laser and receiver of the spectrophotometer must be equipped with the appropriate filters to excite, emit and transmit signals from the fluorescently labeled protein. Protect the transport chamber from light using a black box during experimentation to avoid interference in fluorescence signal. It is best practice to seal the openings on top of the chamber with flexible film to avoid evaporation.

4.8 Collect real-time downstream fluorescence emission readings and ensure a stable signal for at least 5 min.

NOTE: Aliquots from the downstream chamber can be obtained and assessed for fluorescence using a plate reader if a custom-designed spectrophotometer or translucent transport chamber is not available.

4.9 Pipette a pre-calculated volume of stock solution of fluorescently tagged CPCs into the upstream chamber to ensure a final concentration of 3 µM inside the upstream chamber. Observe the downstream fluorescence signal and allow for solute transport to reach a steady increase in slope.

NOTE: A thicker cartilage explant will require longer time to reach steady-state.

4.10 Once the steady state has been reached, take 20 µL from the upstream chamber and add to the downstream chamber ("spike test").

NOTE: A spike in the downstream fluorescence will be observed. This will allow for correlation between fluorescence readings and CPC concentration.

4.11 Collect real-time downstream fluorescence readings.

[Place **Figure 6, Figure 7** and **Figure 8** here]

REPRESENTATIVE RESULTS:

Following equilibrium absorption of CPCs by cartilage, the bath fluorescence decreases when the solute has been uptaken by the tissue. However, if the fluorescence value of the final bath remains similar to the initial, it indicates that there is no/minimal solute uptake. Another confirmation of solute uptake is if the tissue has visibly changed color to the color of the fluorescent dye. The quantitative uptake of solutes in cartilage was determined using the uptake ratio (R_U) after the fluorescence values were converted to concentration using the standard curve. Using the initial bath concentration ($C_{Bath,i}$) and the equilibrium bath concentration (C_{Bath}), the solute concentration inside cartilage ($C_{Cartilage}$) was calculated as follows where V_{Bath}=300 µL:

$$C_{Cartilage} = \frac{(C_{Bath,i} - C_{Bath}) \times V_{Bath}}{Tissue\ Wet\ Weight} \qquad \left[\frac{(\mu M - \mu M) \times \mu L}{mg}\right]$$

Using $C_{Cartilage}$ and C_{Bath}, the uptake ratio was determined using the equation below.

$$R_u = \frac{C_{Cartilage}}{C_{Bath}}$$

Values >>1 indicate enhanced uptake due to charge interactions, whereas values <1 indicate low uptake. For example, larger, neutral solutes such as Neutravidin (60 kDa, pI 7) have shown R_U<1 due to steric hinderance with the cartilage matrix[1], while smaller, neutral solutes are expected to show R_U~1, as they are able to diffuse into the cartilage, reaching equilibrium. In contrast, Avidin (pI 10.5), the positively charged counterpart of Neutravidin, has shown an R_U~180 in cartilage[1]. Further, small-sized CPCs (~2.5-4 kDa) show an R_U up to 400[4]. As shown by **Figure 9**, the uptake ratios showed a charge-dependent response[4].

[Place **Figure 9** here]

In case the fluorescence of the bath has increased after reaching equilibrium, this would indicate that the initial bath concentration of the fluorescently tagged solute was too high. This would cause the emission to be trapped within the solution after excitation via plate reader. To solve this issue, lower the initial bath concentration.

Following confocal imaging, a stack of images was produced with each image showing the depth of penetration of fluorescently tagged CPCs at different layers of cartilage. The image obtained from the center of the cartilage explant showed the furthest depth of penetration compared to any other image throughout the thickness of the explant. Using a software like ImageJ, the stack of images was overlaid to produce one image displaying the average intensity of CPC penetration. These overlaid images provided the best comparison of overall depth of penetration between variously charged drug carriers. A charge-dependent response was observed for CPCs within the tissue **(Figure 10)**. Large neutrally charged carriers (e.g., Neutravidin) will not penetrate much farther than the superficial zone as they lack the ability to use charge interactions to induce binding with the matrix[1]. Similarly, too high a positive charge will be limited to the superficial zone (as shown by CPC+20 even after 24 h)[4], however, this is a result of the carrier being bound too strongly to the matrix; they are unable to unbind from their initial target. An optimally charged drug carrier would, however, be able to penetrate through to the deep zones of cartilage as it can take advantage of the weak and reversible nature of electrostatic interactions (as shown by CPC+14)[4]. This allows for the carrier to bind to its initial target, unbind to move deeper through the matrix, and then bind again to targets further inside the tissue. For example, Avidin (~7 nm diameter, 66 kDa, pI 10.5) binding with negatively charged matrix glycosaminoglycans (GAGs) has a dissociation constant (K_D) of 150 μM, which was considered to be weak enough to enable the reversible binding necessary for full thickness penetration[1]. Despite the weak binding, Avidin showed high retention and uptake in cartilage due to the presence of a high density of negatively charged GAGs (Binding density N_T = 2900 μM)[1]. Further, as shown by CPC+8, full thickness penetration was visible within 4 h, while CPC+14 required 24 h to reach full depth[4]. Thus, multiple time points should be chosen to effectively compare rate of different solutes at penetrating tissue thickness. For a more quantitative understanding of the depth of penetration, the relative intensities of solutes along the thickness of tissue can be obtained using ImageJ.

[Place **Figure 10** here]

If no fluorescent signal has been observed inside the cartilage during imaging, two issues can be present; either the surface area for diffusion was blocked by the epoxy, or the initial bath concentration was too low to produce a fluorescent signal. To fix these issues, remove excess epoxy from the cartilage surfaces and increase solute concentration.

Non-equilibrium diffusion transport experiments resulted in a curve as shown in **Figure 11**. The initial part of the curve represents solute diffusion through cartilage as solute-matrix binding interactions take place. With increased charge of the carrier, stronger matrix binding occurred which will result in a longer time for solutes to reach the downstream chamber. Once solutes penetrated through the depth of the cartilage and reached the downstream chamber, an increase in slope of the curve was observed as the fluorescence reading increased over time. This second part of the curve reached a steady slope, representing steady-state diffusion. A tangential line was drawn at the steady-state slope to determine the time it takes to reach steady-state diffusion (τ_{Lag}), marked by the x-intercept. The effective diffusivity (D_{EFF}), the diffusion rate of CPCs while there are binding interactions present in cartilage, was calculated using the τ_{Lag} and

explant thickness (L) as follows:

$$\tau_{Lag} = \frac{L^2}{6D_{EFF}}$$

Following transfer of 20 µL of solution from the upstream to downstream chamber, a spike in fluorescence was observed; the resulting stabilized fluorescence intensity was used for correlation to the concentration. The concentration of CPCs in downstream (C_D) normalized to the upstream concentration (C_U) was then plotted against time. The slope of this curve was used to estimate the steady-state diffusion rate when all binding sites in cartilage are occupied (D_{SS}) as shown below. This value was inclusive of the partitioning coefficient. Here, ϕ, V_D and A represent cartilage porosity (~0.8), downstream chamber volume (2 mL) and cross-sectional area of cartilage (0.1257 cm^2), respectively. Representative D_{EFF} and D_{SS} values calculated from non-equilibrium transport experiments for CPCs can be found in **Table 1**.

$$\frac{\partial}{\partial t}\left(\frac{C_D}{C_U}\right) \cong \frac{\Phi D_{ss} A}{L V_D}$$

[Place **Figure 11** here]

If the downstream fluorescence fails to stabilize prior to the addition of fluorescently tagged peptide to the upstream chamber, it is likely that there is solute residue stuck on the walls from a previous experiment. In this case, disassemble the chamber and wash with soap and sonicate. If there is an increase in the downstream fluorescence immediately following the addition of fluorescently tagged peptide to the upstream chamber, this could indicate that leakage is present. This would require reassembly of the transport chamber and re-testing for leaks. If the downstream fluorescence signal reaches a plateau as opposed to a steady-state increase, it indicates a possible loss of concentration in the upstream chamber, likely caused by solutes sticking to the walls of the chamber. Addition of 0.005% w/v bovine serum albumin (BSA) to the upstream chamber can help prevent sticking.

FIGURE AND TABLE LEGENDS:

Figure 1: Charge based intra-cartilage delivery of CPCs. Intra-articular injection of CPCs into the knee joint space. Electrostatic interactions between positively charged CPCs and negatively charged aggrecan groups enable rapid and full depth penetration through cartilage. This figure has been modified from Vedadghavami et al[4].

Figure 2: Custom-designed slicing fixture. Design parameters of stainless steel slicing fixture used for slicing cartilage explants of 3 and 6 mm diameter. Plastic inserts of varying thickness were placed inside wells to adjust the thickness of sliced explants. Stainless steel cylindrical pin of <1 mm diameter was used to push explant out of fixture. All numerical values are presented in mm.

Figure 3: Schematic of equilibrium uptake experiments. Cartilage explants (3 mm dia. x 1 mm

thick) were placed in individuals wells in a 96-well plate containing fluorescently tagged CPC solution. After 24 h CPCs were uptaken by the cartilage, thereby reducing the fluorescence of the surrounding bath.

Figure 4: Custom-designed 1-D transport chamber. Design parameters of PMMA 1D transport chamber with 6 individual wells. All numerical values are presented in mm.

Figure 5: Schematic of depth of penetration studies. Cartilage explants (6 mm diameter x 1 mm thickness) were cut in half and fixed to the center of 1-D diffusive transport wells. Fluorescently tagged CPC solution was added to the side of the well in contact with the superficial zone (SZ) of cartilage. 1x PBS-PI was added to the side of the well in contact with the deep zone (DZ) of cartilage. Following diffusion, a cross-section of cartilage (3 mm x 1 mm) was imaged using confocal microscopy. This figure has been modified from Vedadghavami et al.[4] and Bajpayee et al.[3]

Figure 6: Custom-designed non-equilibrium diffusion transport chamber. Design parameters of PMMA non-equilibrium diffusion transport chamber. The chamber must be translucent as to not interfere with fluorescence readings. The complete transport chamber consisted of two identical halves of the fixture shown. Two cylindrical stainless-steel pins (∼2.94 mm diameter, ∼18 mm long) were required to ensure alignment and complete closure of the halves of the chamber. Four identical slots for 6-32 thread screws were made in each corner of the chamber for screw tight assembly. All numerical values are presented in millimeters.

Figure 7: Assembly of non-equilibrium diffusion transport chamber. Design parameters of (**A**) black PMMA inserts and (**B**) large and small rubber gaskets. Thickness of rubber gaskets was adjusted to ensure tight closure of the chamber. All numerical values are presented in mm. (**C**) Schematic showing the order of assembly for two halves of transport chamber with cartilage explant placed in the center. SZ indicates superficial zone of cartilage which was facing the upstream chamber.

Figure 8: Schematic of non-equilibrium diffusion experiments. Cartilage explants (6 mm diameter x 1 mm thickness) were placed in the center of the transport chamber with the superficial surface facing the upstream chamber. Both up and downstream sides of the chamber were filled with 1x PBS-PI and mixed using a mini stir bar. With a laser pointed towards the downstream chamber to collect fluorescent readings, fluorescently tagged CPC solution was added to the upstream chamber.

Figure 9: Representative results of equilibrium uptake of CPCs in cartilage. CPCs of varying net charge (+8, +14 and +20) and their respective uptake ratios in cartilage revealed that uptake does not increase monotonically with increasing charge. This figure has been modified from Vedadghavami et al.[4]

Figure 10: Representative results from depth of penetration studies in cartilage. CPCs of varying net charge (+8, +14 and +20) and their respective depth of penetration through cartilage revealed

weak-reversible binding as seen by CPC+8 and CPC+14 is key for full depth penetration. However, too strong binding as seen for CPC+20 impeded full thickness penetration. This figure has been modified from Vedadghavami et al.[4]

Figure 11: Representative results from non-equilibrium diffusion studies through cartilage. CPC+8 diffusion curve, plotted as the downstream concentration (C_D) normalized to the upstream concentration (C_U), against time. A tangential line drawn at the steady-state slope (blue) crosses the x-axis at τ_{Lag}, which was used to calculate D_{EFF}. The slope of the tangent was used to calculate D_{SS}. The spike test (gray) represents the stabilized concentration in the downstream chamber following transfer of 20 µL CPC solution from upstream to downstream, used for normalizing downstream concentration. This figure has been modified from Vedadghavami et al.[4]

Table 1: Representative D_{EFF} and D_{SS} values for CPC transport through cartilage. This table has been modified from Vedadghavami et al.[4]

DISCUSSION:

The methods and protocols described here are significant to the field of targeted drug delivery to negatively charged tissues. Due to the high density of negatively charged aggrecans present in these tissues, a barrier is created, thus preventing drugs from reaching their cellular target sites which lie deep with the matrix. To address this outstanding challenge, drugs can be modified to incorporate positively charged drug carriers which can enhance the transport rate, uptake and binding of drugs within tissue[1,3,4,12–15]. As shown here with the developed methods, the transport of positively charged drug carriers can be characterized to determine the equilibrium uptake, depth of penetration and non-equilibrium diffusion rate. We have successfully designed three separate experimental setups which can be utilized for assessing the transport through cartilage explants.

For the successful characterization of transport, critical steps in the procedure need to be followed. The use of protease inhibitors (PIs) in all solutions is critical for accurately characterizing intra-cartilage transport of CPCs through cartilage as they function to prevent enzymatic digestion of proteins in tissue[16]. Therefore, if not used, cartilage matrix components such as aggrecans and collagen can begin to degrade and secrete into the surrounding bath during experimentation. This can greatly lower the FCD of cartilage, reducing the number of charge-based binding sites in the cartilage matrix. The resulting tissue would no longer be representative of healthy cartilage. Conversely, the experiments presented can also be used to evaluate the transport of CPCs through arthritic cartilage where the aggrecan content is much lower as seen in OA[16]. Using trypsin or Chondroitinase ABC to digest cartilage explants, the aggrecan density can be controlled, thereby allowing for the evaluation of transport and drug delivery in a diseased state. In this case, charge-based binding may be compromised, while other types of interactions such as hydrogen bonds and hydrophobic interactions synergistically enhance intra-cartilage binding and retention[4].

Maintaining hydration of the cartilage explant is the key during sample preparation and experimentation. Dehydration via exposure to air for greater than 6 minutes has been shown to

induce irreversible damage to articular cartilage[17]. As a result, unexpected changes in the transport of CPCs may occur. Similarly, evaporation of CPC baths can result in explant dehydration; this can be prevented by sealing with a flexible film. However, bath evaporation can not only cause explant dehydration, but it can also cause a change in CPC bath concentration, resulting in false fluorescent readings. Further it is important to note that depth of penetration studies require thin cross-sections (~100 μM) of cartilage to be imaged. This is a technique which requires practice so that slices of uniform thickness can be obtained. It is also critical for non-equilibrium diffusion experiments, that the transport chamber be translucent so that real-time fluorescence measurements can be obtained with the custom-designed spectrophotometer. However, as an alternative, aliquots from the downstream chamber can be obtained and assessed for fluorescence using a plate reader or other spectrophotometric reader.

The methods presented here are of great significance as they provide a bench-scale method for characterizing drug carrier transport through the cartilage in order to better predict in vivo drug retention and long-term biological efficacy. Recently, a finite element framework for computational fluid dynamics was implemented for measuring solute transport through porous media[18]. Arbabi et al. have used finite element analysis in combination with experimental data obtained from micro-CT imaging to measure diffusion rates of negatively charged contrast agent, ioxaglate in cartilage[19,20]. Further, using a multi-zone, multi-phasic model, the diffusion coefficients of ioxaglate in different zones of cartilage were measured along with the FCD of each zone. While micro-CT imaging can only be used with contrast agents, our experimental setup allows for the characterization of transport of all drugs and drug carriers that can be fluorescently labeled. However, the advanced computation modeling used by Arbabi et al. provides a more comprehensive analysis of solute transport behavior and may be applied to our experimental methods[19,20].

A limitation of the presented method is that the experimental setup for each solute transport experiment does not fully encompass the in vivo environment. Biological responses and mechanical and dynamic forces that occur within the natural joint are not simulated here. To incorporate these forces, the transport chamber can be modified with a piston to simulate convective flow patterns that occur during activities as walking and running. However, while convective flow can increase uptake by 2-fold, uptake due to electrostatic interactions can increase 100-400x. Thus, the experimental setups presented here provide a good estimate for charge-based transport and uptake[21]. Further, since the knee joint naturally contains synovial fluid, it can be used in the bath solutions for transport experiments instead of 1x PBS-PI. It is estimated that the uptake of cationic carriers in cartilage would decrease in synovial fluid compared to in 1x PBS due to the presence of hyaluronan chains with negatively charged carboxyl groups in synovial fluid. It is possible that cationic carriers competitively bind with the hyaluronan chains of the synovial fluid in addition to the GAGs of cartilage. However, the density of negatively charged groups is significantly higher in cartilage compared to synovial fluid, due to the presence of both negatively charged carboxylated hyaluronan chains and sulfated GAGs in cartilage[13]. Thus, although the uptake in cartilage in presence of synovial fluid will be lower than in 1x PBS, it is still expected to maintain high intra-cartilage uptake. In vivo, Avidin has shown high intra-cartilage uptake in both rat and rabbit cartilage in presence of synovial fluid[14,22]. Further, Avidin

has shown high uptake and retention in cartilage up to 2 weeks following intra-articular injection in a rabbit anterior cruciate ligament transection model[23].

Use of bovine cartilage in this system allows for a more accurate representation of drug penetration through cartilage due to its similarities to human cartilage in terms of thickness (~1.5-2 mm)[24,25]. Transport of solutes through the cartilage can vary with thickness; drug carriers may require fewer binding interactions to fully penetrate through mice or rat cartilage which are much thinner, however can significantly be hindered from penetrating deeper in thicker human cartilage[1]. Further, although these experiments were designed to characterize solute transport within the cartilage, these methods can be modified and applied to other negatively charged tissues such as meniscus, cornea and vitreous humor of the eye, and the nucleus pulposus of intervertebral disks. The methodologies of experiments designed here are advantageous as the dimensions of fixtures and transport chambers can be adapted according to the size and species of tissue. The impact of these methods is widespread, limited not only to drug carriers but also for the evaluation of transport of drugs and drug-drug carrier conjugates.

ACKNOWLEDGMENTS:

This work was funded by the United States Department of Defense through the Congressionally Directed Medical Research Programs (CDMRP) under contract W81XWH-17-1-0085, and the National Institute of Health R03 EB025903-1. AV was funded by the College of Engineering Dean's Fellowship at Northeastern University.

DISCLOSURES:

The authors have nothing to disclose.

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658 *Traumatology*. **19** (3), 142–146 (2006).
659

Figure 1

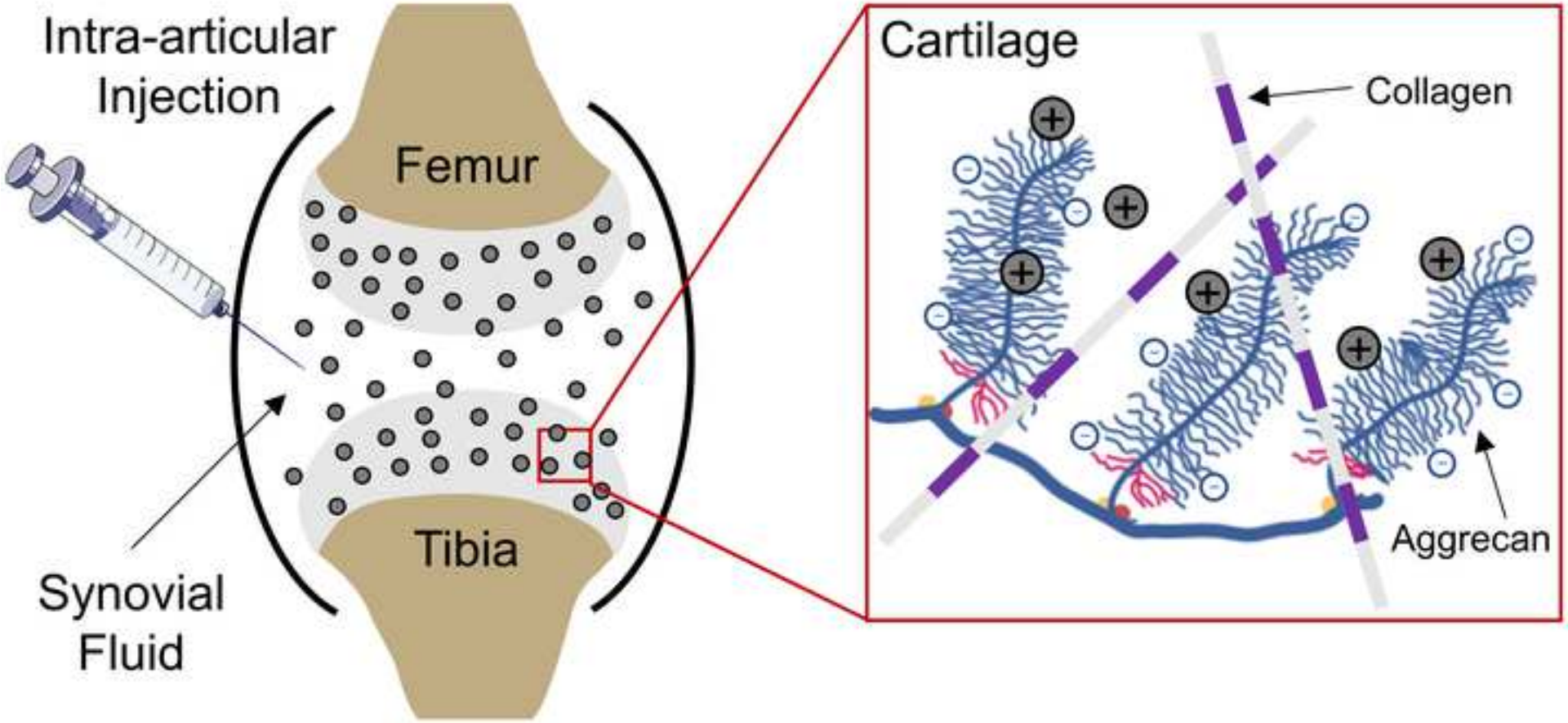
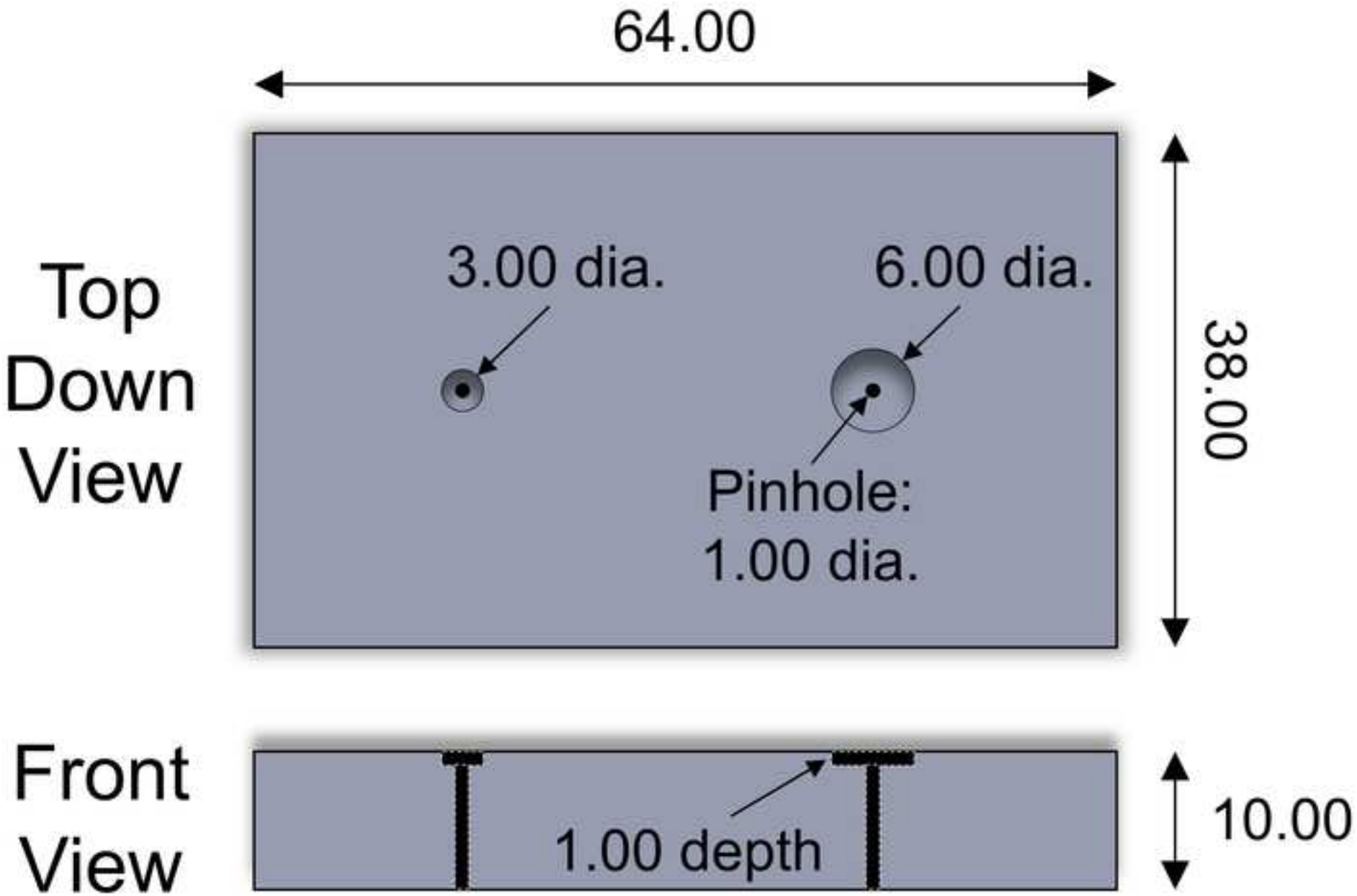


Figure 2_Revised



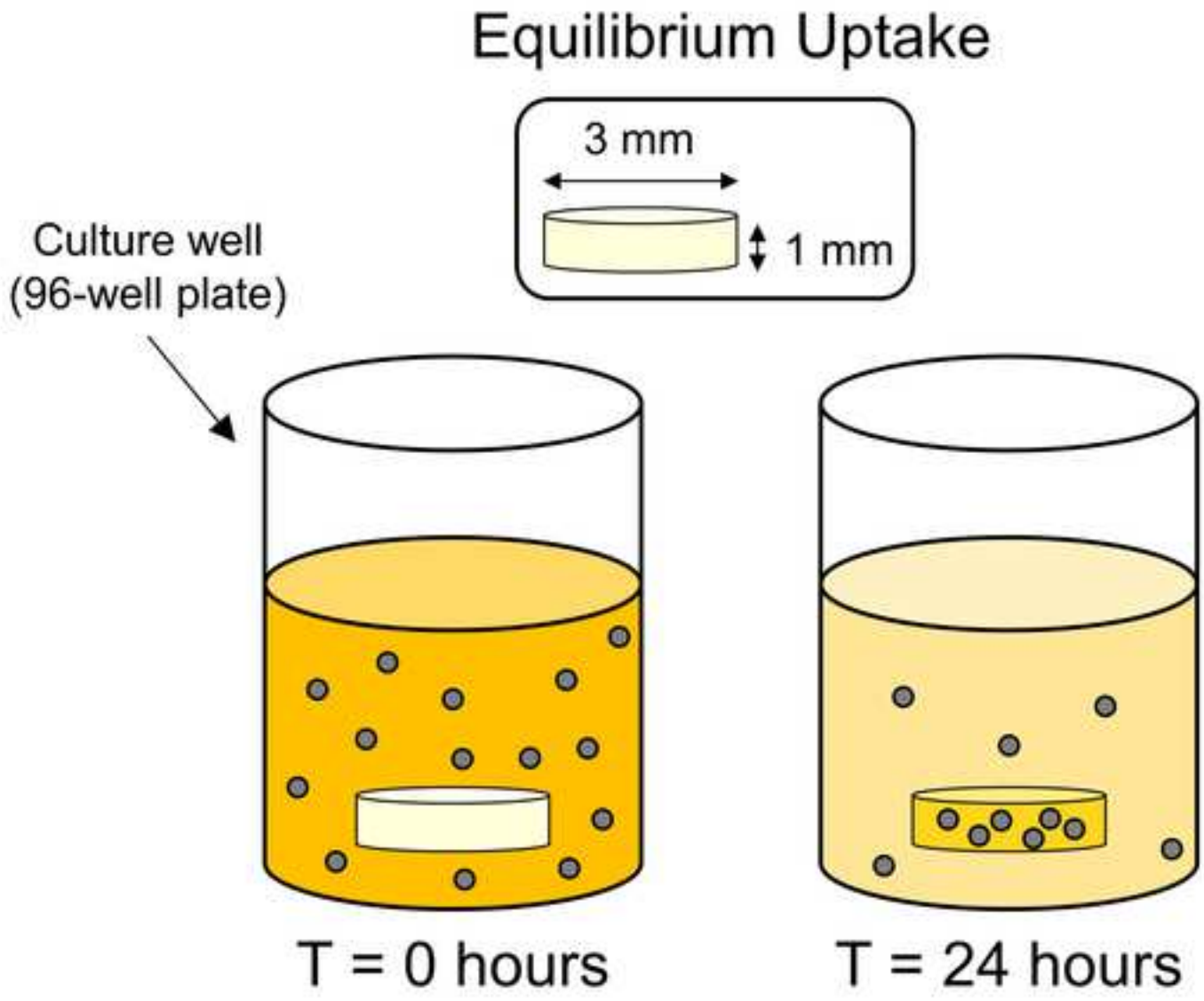


Figure 4_Revised

Click here to access/download;Figure;Figure 4_Revised.tif

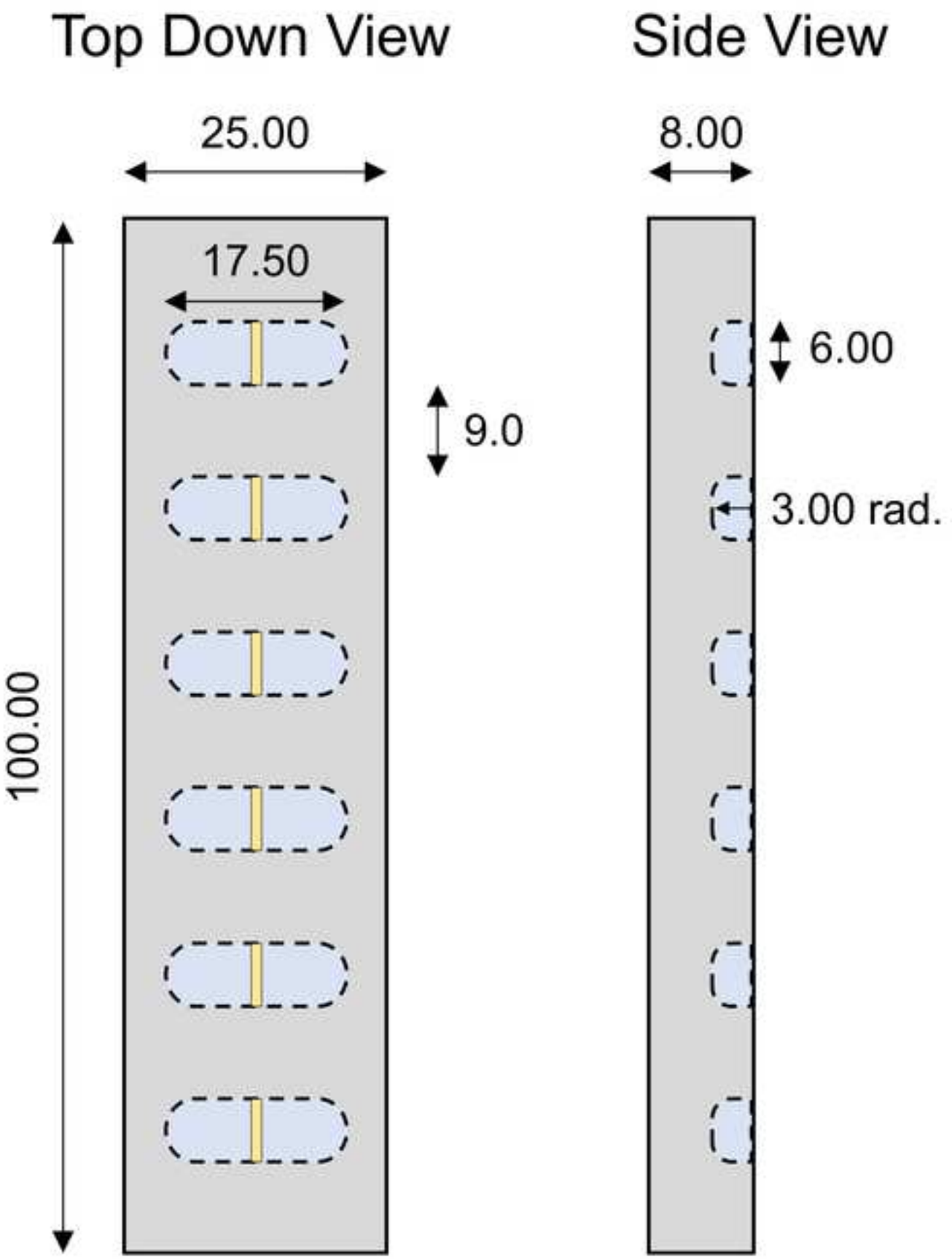


Figure 5_Revised

Click here to access/download;Figure;Figure 5_Revised.tif

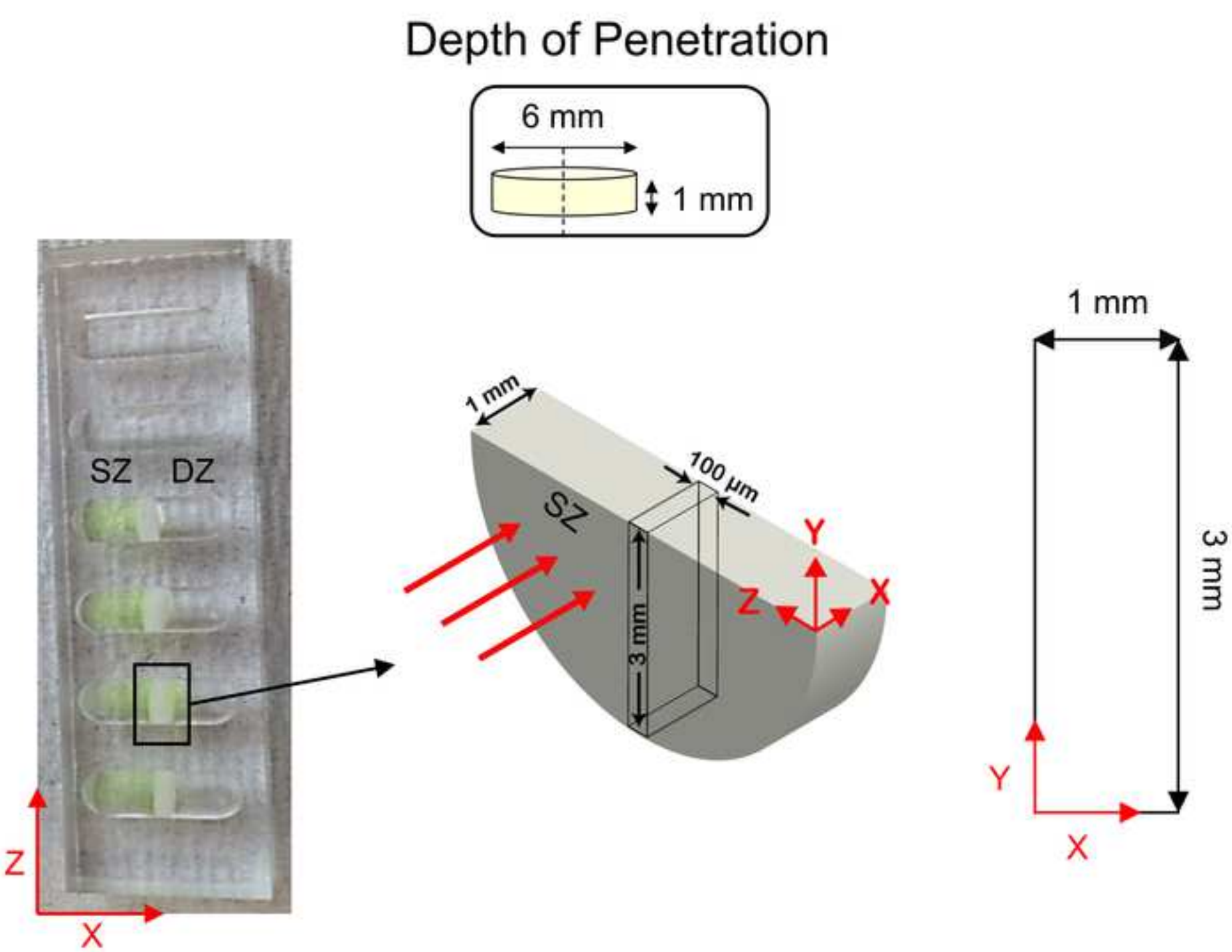


Figure 6_Revised

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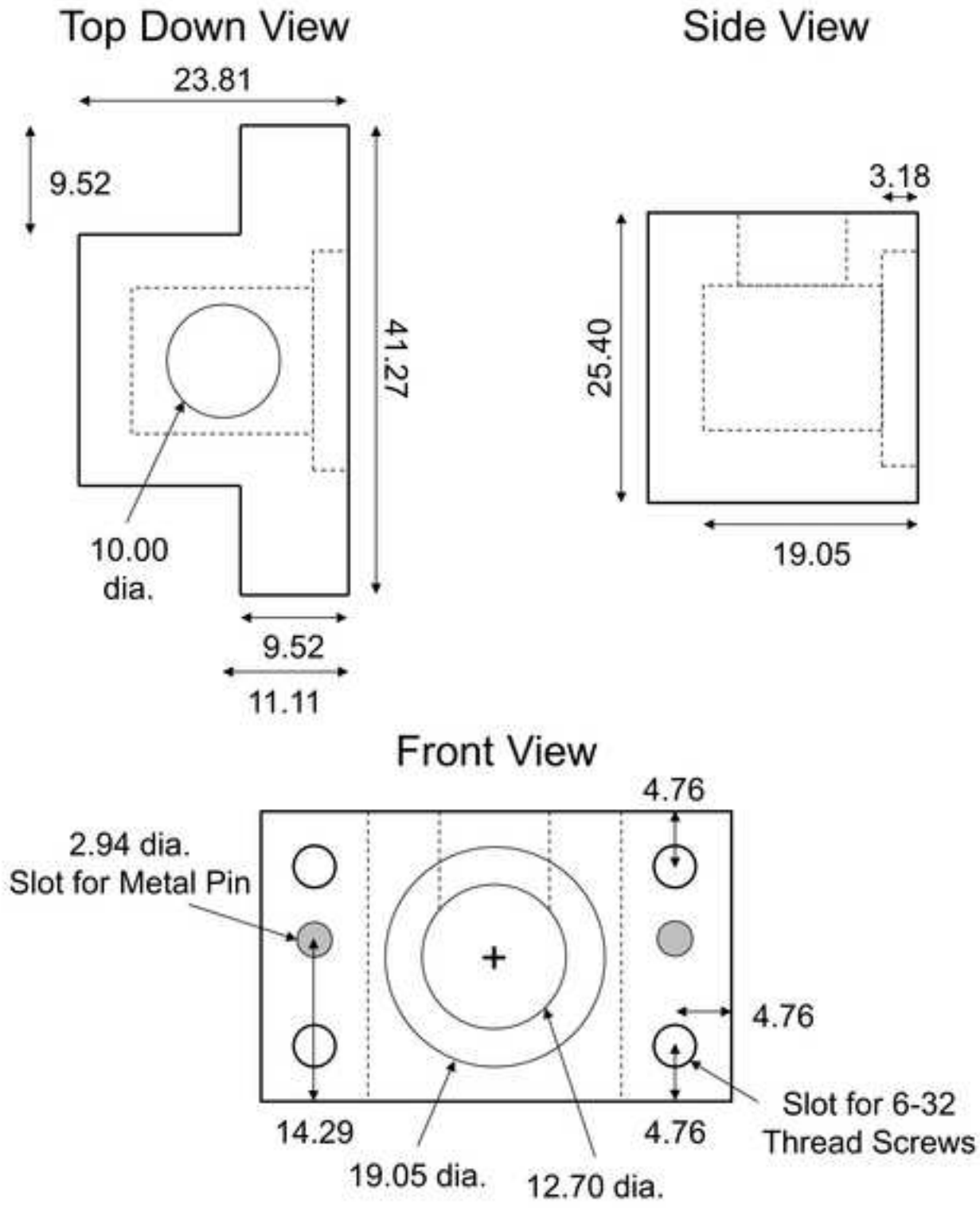

Figure 7_Revised

Click here to access/download;Figure;Figure 7_Revised.tif

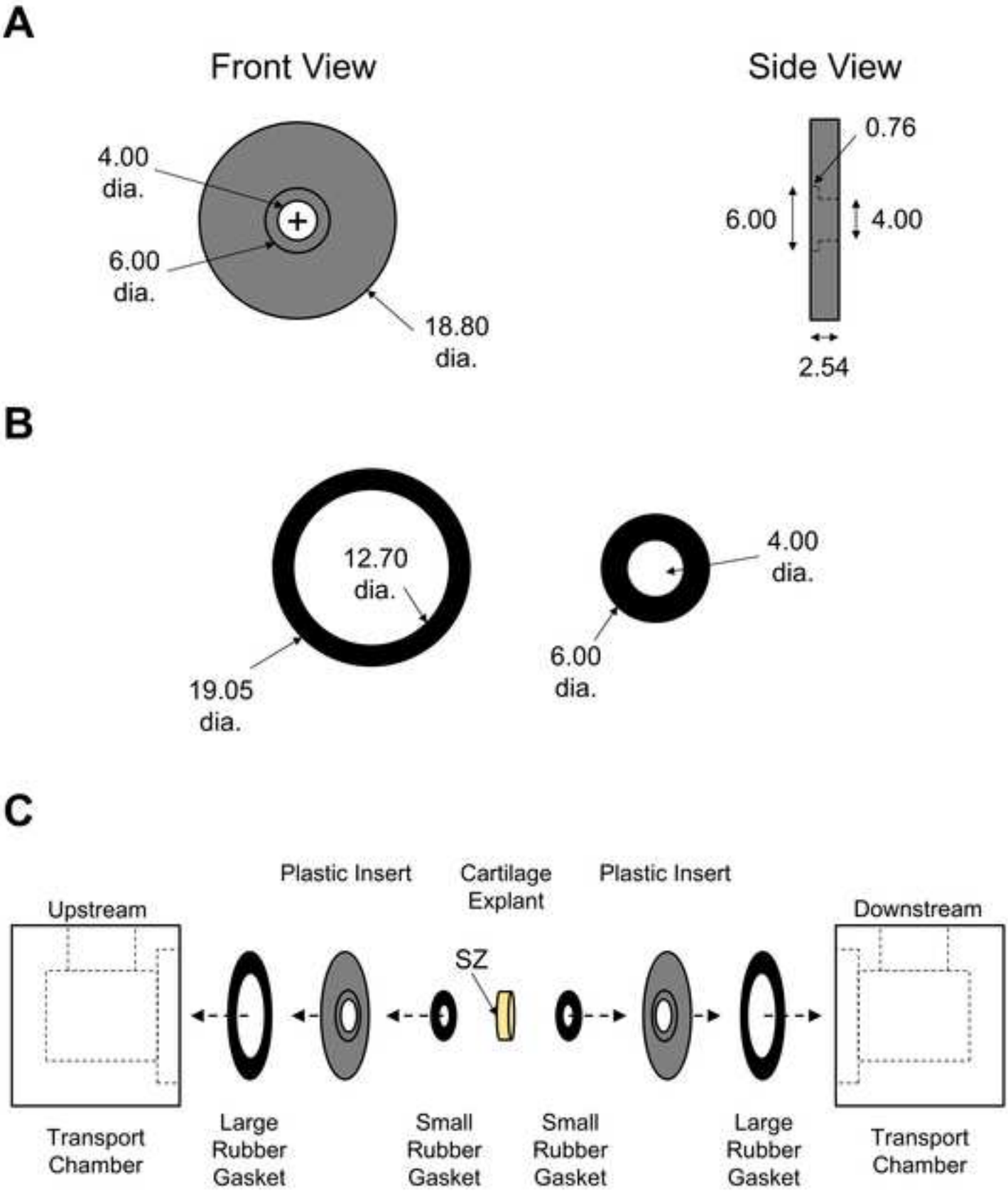

Figure 8_Revised

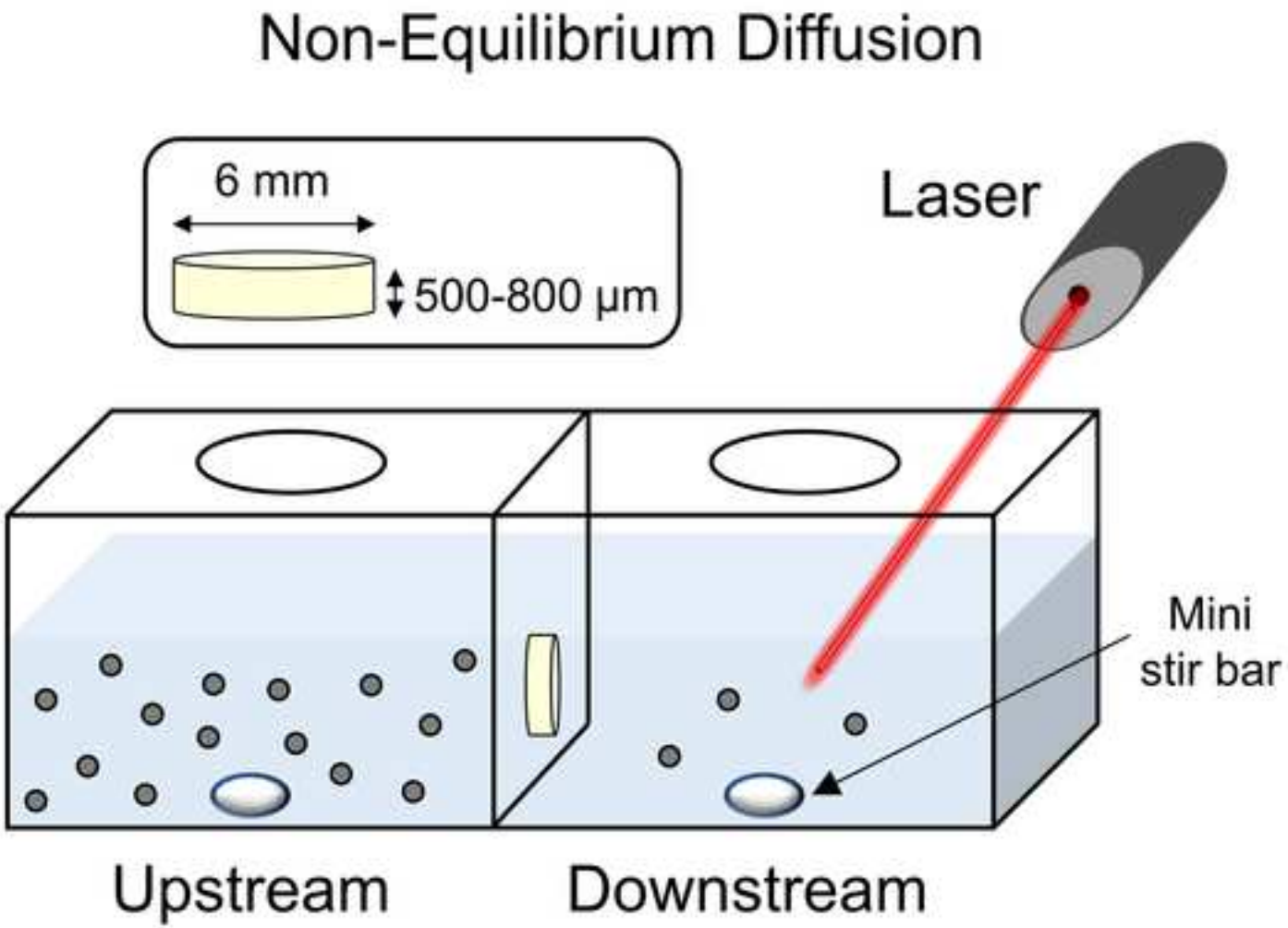

Figure 9_Revised

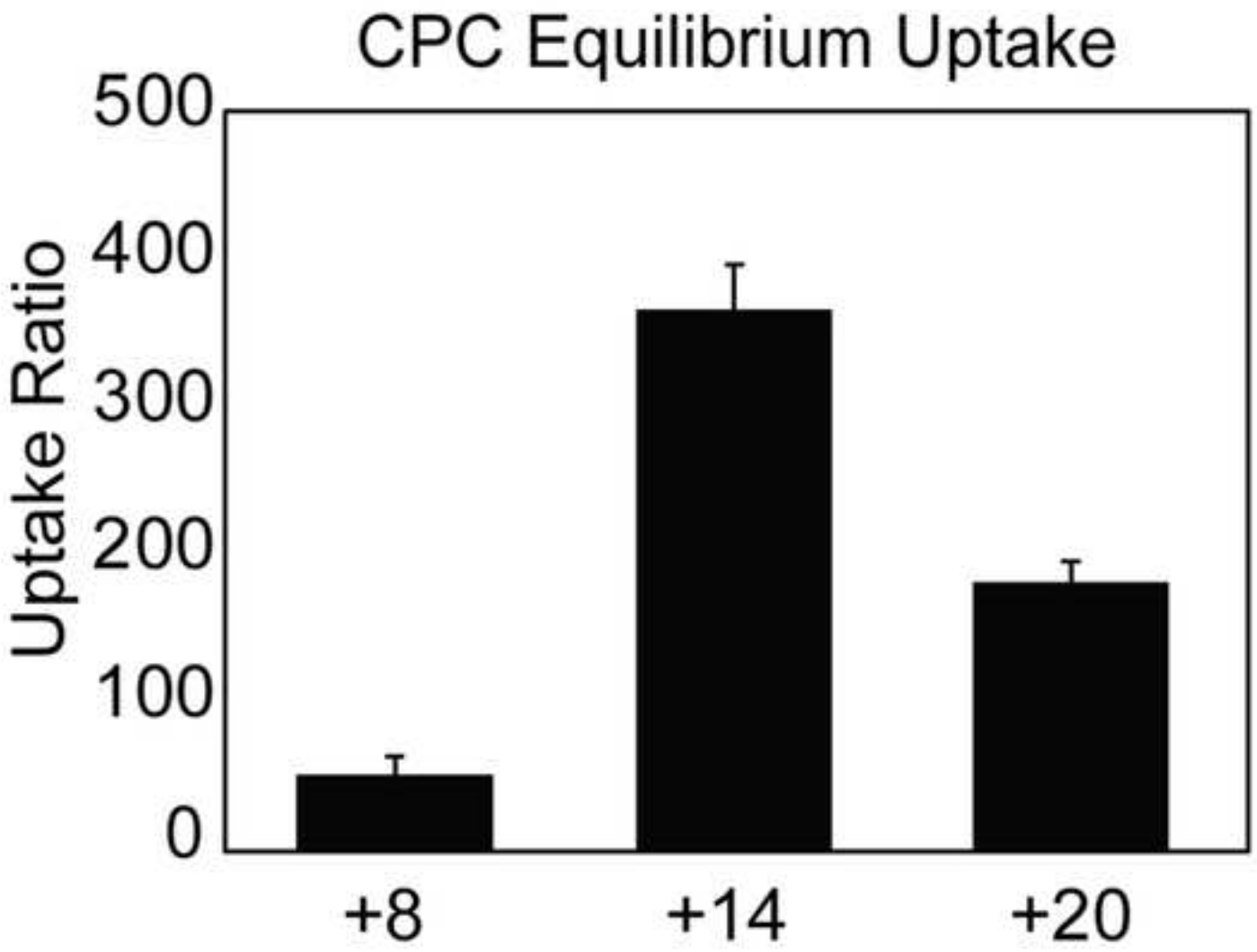

Figure 10_Revised

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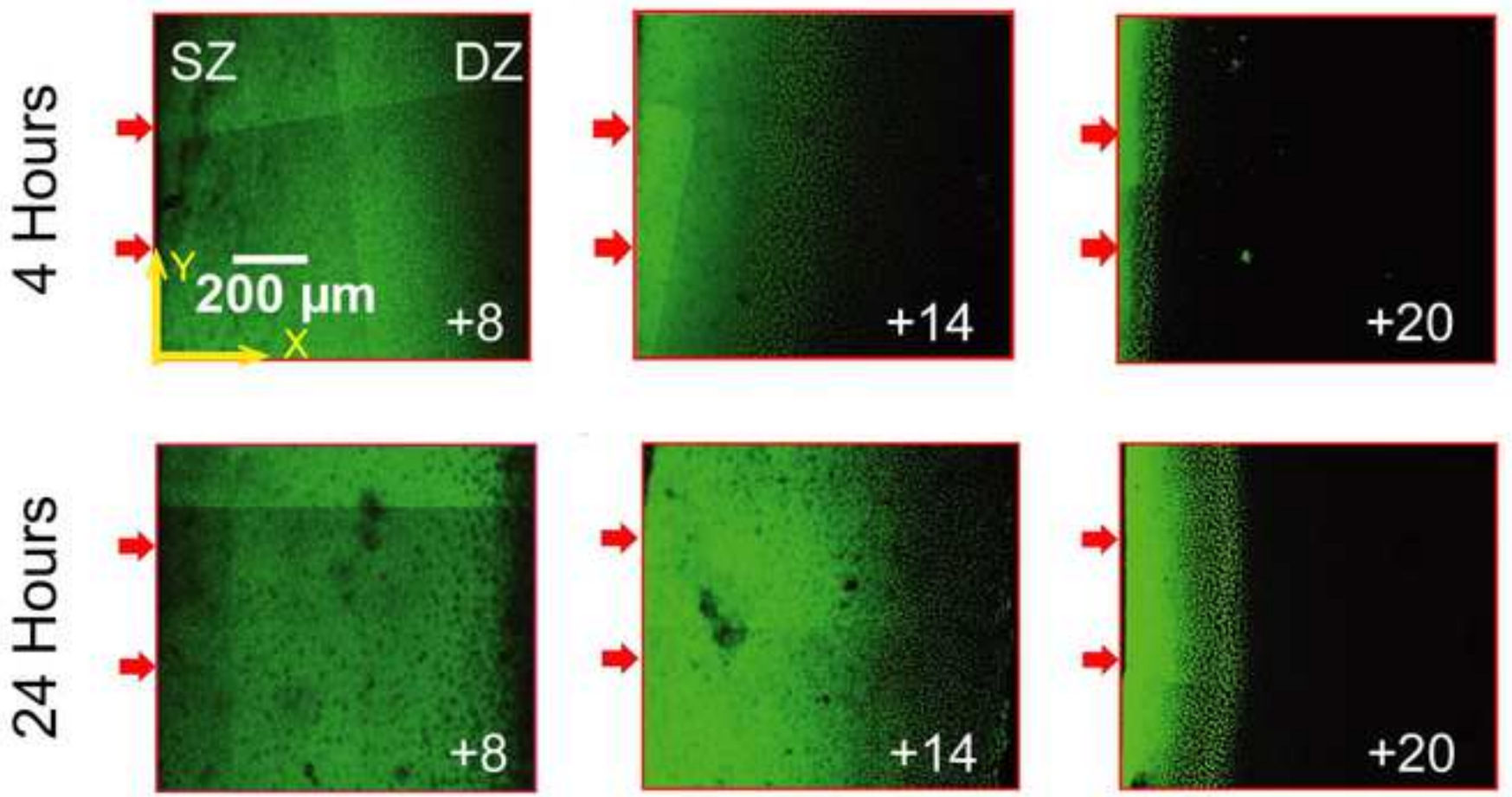

Figure 11_Revised

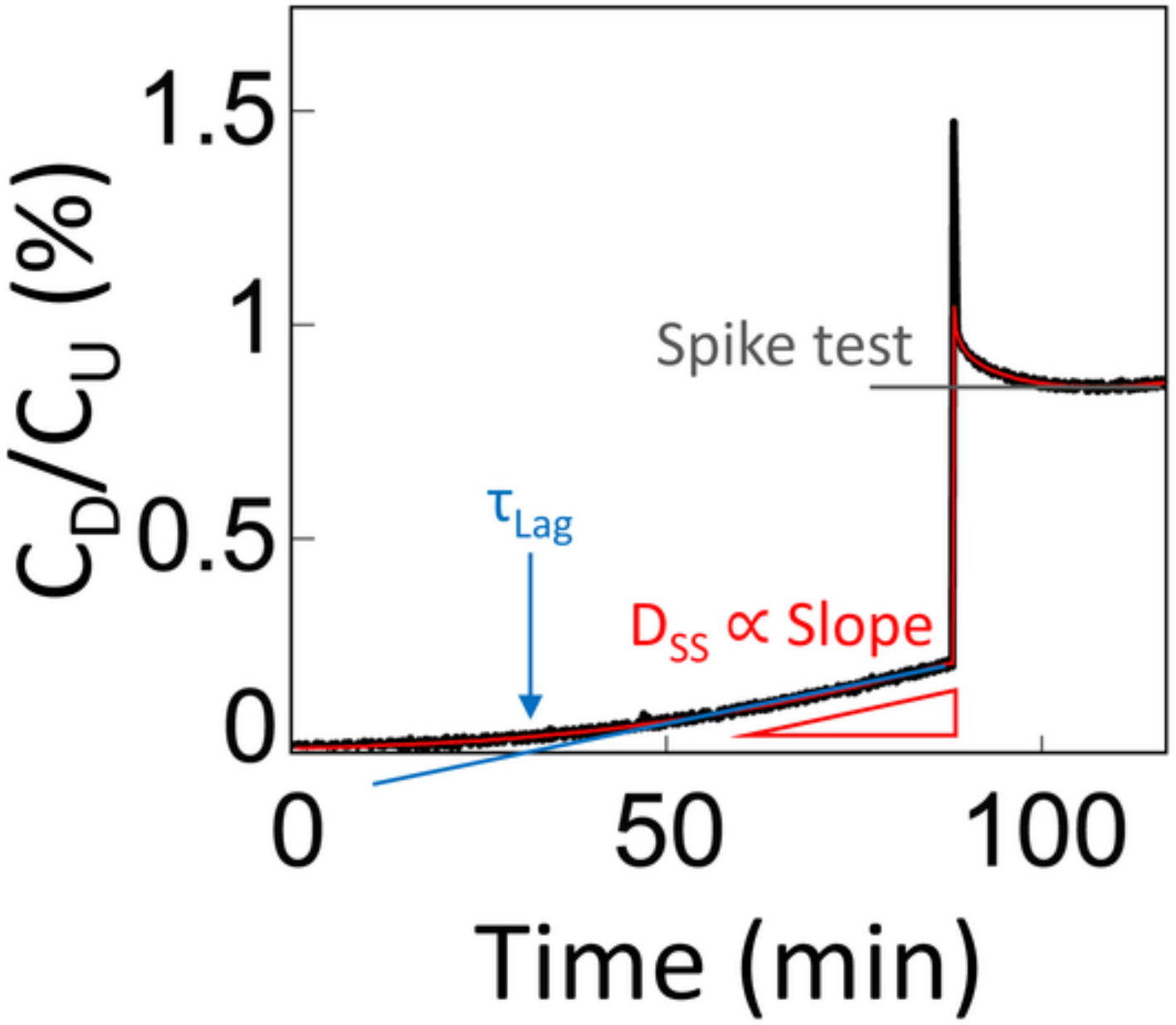

Table 1

CPC	D_{EFF} (cm^2/s)	D_{SS} (cm^2/s)
CPC+8	$1.7 \pm 0.4 \times 10^{-7}$	$5.8 \pm 0.0 \times 10^{-5}$
CPC+14	$9.8 \pm 0.2 \times 10^{-8}$	$2.6 \pm 1.2 \times 10^{-5}$
CPC+20	$4.7 \pm 0.1 \times 10^{-8}$	$1.4 \pm 0.9 \times 10^{-5}$

Name of Material/ Equipment	Company	Catalog Number
316 Stainless Steel SAE Washer	McMaster-Carr	91950A044
96-Well Polystyrene Plate	Fisherbrand	12566620
Acrylic Thick Gauge Sheet	Reynolds Polymer	N/A
Antibiotic-Antimycotic	Gibco	15240062
Bovine Cartilage	Research 87	N/A
Bovine Serum Albumin	Fisher BioReagents	BP671-1
CPC+14	LifeTein	LT1524
CPC+20	LifeTein	LT1525
CPC+8	LifeTein	LT1523
Delicate Task Wipers	Kimberly-Clark Professional	34155
Dermal Punch	MedBlades	MB5-1
Economy Plain Glass Microscope Slides	Fisherbrand	12550A3
Flat Bottom Cell Culture Plates	Corning Costar	3595
Flexible Wrapping Film	Bemis Parafilm M Laboratory	1337412
Gold Seal Cover Glass	Electron Microscopy Sciences	6378701
Hammer-Driven Hole Punch	McMaster-Carr	3427A15
Hammer-Driven Hole Punch	McMaster-Carr	3427A19
Laser	Chroma Technology	AT480/30m
Low-Strength Steel Hex Nut	McMaster-Carr	90480A007
LSM 700 Confocal Microscope	Zeiss	LSM 700
Micro Magnetic Stirring Bars	Bel-Art Spinbar	F37119-0007
Multipurpose Neoprene Rubber Sheet	McMaster-Carr	1370N12
Non-Fat Dried Bovine Milk	Sigma Aldrich	M7409

Petri Dish	Chemglass Life Sciences	CGN1802145
Phosphate-Buffered Saline	Corning	21-040-CMR
Plate Shaker	VWR	89032-088
Protease Inhibitors	Thermo Scientific	A32953
Razor Blades	Fisherbrand	12640
R-Cast Acrylic Thin Gauge Sheet	Reynolds Polymer	N/A
RTV Silicone	Loctite	234323
Scalpel	TedPella	549-3
Signal Receiver	Chroma Technology	ET515lp
Snap-Cap Microcentrifuge Tubes	Eppendorf	22363204
Spatula	TedPella	13508
Synergy H1 Microplate Reader	Biotek	H1M
Zinc-Plated Alloy Steel Socket Head Screw	McMaster-Carr	90128A153

Comments/Description
For number 5 screw size, 0.14" ID, 0.312" OD
Black
For non-equilibrium diffusion and 1-D diffusion transport chamber
100x
2-3 weeks old, femoropatellar groove
Custom designed peptide
Custom designed peptide
Custom designed peptide
3, 4 and 6 mm
Clear, 96 well
1.5, 18x18 mm
1/2" Diameter
3/4" Diameter
Spectrophotometer Laser Light
6-32 Thread size
7x2 mm
1/32" Thickness

150 mm diameter
1x

Black transport chamber inserts
Epoxy, Non-corrosive, clear
#10, #11 blades

Spectrophotometer Laser Signal Receiver

1.5 mL

6-32 Thread size, 1" Long

Editorial Comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

   Authors' Response: We have now thoroughly proofread the manuscript and have corrected any spelling or grammatical errors.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

   Authors' Response: Paragraph indentation has now been set to 0 and all other indentations have been corrected. We have now included a single line space between each step, substep and note in the protocol section. Calibri 12 points font is used throughout the manuscript.

3. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Presented here is a protocol …"

   Authors' Response: We rephrased the Summary to describe the protocols and their application as follows.

   "Presented here is a protocol for determining equilibrium uptake, depth of penetration and non-equilibrium diffusion rate of cationic peptide carriers in cartilage. Characterization of transport properties is critical for ensuring an effective biological response. These methods can be applied for designing optimally charged drug carriers for targeting negatively charged tissues."

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

   Authors' Response: Thank you for highlighting this. We have modified the protocol to ensure all steps are written in the imperative tense. Text not written in the imperative tense have been added as a note. All instances of "could be," "should be," and "would be" have been removed from the protocol section.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
   For example: Kimwipes, Eppendorf, etc.

   Authors' Response: All commercial language has been removed from the manuscript and generic terms have been used instead. The Table of Materials and Reagents has been updated accordingly.

6. The Protocol should contain only action items that direct the reader to do something.

Authors' Response: The protocol has been edited to remove phrasing that does not direct the reader to perform an action.

7. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections.

   Authors' Response: The protocol is written with discrete steps and we have ensured that there are no large paragraphs of text between sections.

8. Please include volume and concentrations for all the solutions used.

   Authors' Response: Thank you for your comment. We have now added concentrations and volumes for each solution used in the protocol.

9. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

   Authors' Response: Thank you. The protocol has now been adjusted to meet this requirement.

10. Please ensure you answer the "how" question, i.e., how is the step performed?

    Authors' Response: We have made the appropriate changes throughout the protocol to ensure the "how" has been addressed. We have included additional figures to aid in this process.

11. What kind of tissue explants are used? How do you get it? Human/animal origin? Please include ethics statement accordingly before the start of the protocol.

    Authors' Response: Bovine cartilage explants were used for these experiments, however, the experimental setups are compatible for use with most cartilage species including human. Bovine cartilage explants are ideal for evaluation of solute transport because of their similarity in composition and thickness to human cartilage, however explants obtained from other large animals such as horses and pigs can be used as well *[Bajpayee et al. Nature Reviews Rheumatology, 2017]*. The bovine cartilage explants used in our experiments were extracted using dermal punches from the femoropatellar grooves of bovine knee joints supplied from Research 87 (Boylston, MA, USA). Using the slicing fixture shown in Figure 2, explants were sliced to 1 mm thickness, inclusive of the superficial zone. We have added a section ("Cartilage Explant Extraction") to the protocol to describe this process.

12. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

    Authors' Response: We have now highlighted filmable content of the protocol.

13. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Authors' Response: We have received explicit copyright permission for use of figures from the following publications:

Vedadghavami, A., Wagner, E. K., Mehta, S., He, T., Zhang, C., & Bajpayee, A. G. (2019). Cartilage penetrating cationic peptide carriers for applications in drug delivery to avascular negatively charged tissues. *Acta biomaterialia*, *93*, 258-269.

Bajpayee, A. G., Wong, C. R., Bawendi, M. G., Frank, E. H., & Grodzinsky, A. J. (2014). Avidin as a model for charge driven transport into cartilage and drug delivery for treating early stage post-traumatic osteoarthritis. *Biomaterials*, *35*(1), 538-549.

14. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
    a. Critical steps within the protocol
    b. Any modifications and troubleshooting of the technique
    c. Any limitations of the technique
    d. The significance with respect to existing methods
    e. Any future applications of the technique

Authors' Response: Thank you for detailing these requirements. We have discussed critical steps involving explant hydration, use of protease inhibitors and prevention of solute evaporation to ensure accurate representation of solute transport through cartilage. We have also indicated the potential outcomes should these critical steps not be followed and provided methods for troubleshooting these issues if they occur. In addition, we have now mentioned the importance of using a translucent transport chamber for non-equilibrium diffusion studies in order to obtain real-time fluorescence measurements. However, in the case that this is not possible, we have provided an alternative method by taking aliquots from the downstream chamber for fluorescence measurements using a plate reader instead. We have also addressed the limitations of the protocol as the transport experiments used do not fully encompass the *in vivo* environment. The convective flow present within the joint has not been included here, however the transport chambers can be modified to incorporate fluid flow using a piston. Uptake due to convective flow can increase by 2-fold *[Sampson et al. Journal of Biomechanics, 2019]*, however due to electrostatic interactions, uptake increases 100-400x *[Vedadghavami et al. Acta Biomaterialia, 2019; Bajpayee et al. Nature Reviews Rheumatology]*; therefore, even though convective flow has not been included here, our experimental setups will provide a very good estimate for charged-based transport and uptake. Further, our model has used 1x PBS instead of synovial fluid as the medium for transport experiments. We have addressed this in the Discussion section and detailed the possible changes in transport properties as a result of charge-based binding between positively charged drug carriers and negatively charged synovial fluid hyaluronan chains. Further, we have added discussion on the limitations of our analytical models and presented comparisons to pre-existing methods. We have now included more details on the significance of our methods and commented on how our methods can be applied to a variety of cartilage sizes and other negatively charged tissues.

15. Please include a completer list of materials, reagents, equipment used in the table of materials. Please sort the table in alphabetical order.

Authors' Response: We have now included more complete list of materials, reagents and equipment used in the table of materials. It has also been sorted in alphabetical order.

Reviewer #1 Comments

Manuscript Summary:

In this article, the authors describe a protocol to evaluate the transport of cationic peptide carriers into cartilage, with the goal of developing drug carriers that exploit electrostatic interactions with the tissue. In recent years, there has been increased interest in the design of drug delivery systems that can target or improve bioavailability of therapeutics to cartilage - as such, this article is informative and timely. Overall the paper is clear and well-written. Some additional details are needed in the protocol and representative results sections as well as discussion on limitations.

Authors' Response: We appreciate the reviewer's interest and are encouraged by their comments. We have addressed their concerns below.

Major Concerns:

In several parts of the protocol, the authors mention custom-made items (for example, the custom-designed 1-dimensional transport chamber and custom-designed slicing fixture). Please provide the specifications for these such that they can be reproduced if needed.

Authors' Response: Thank you for informing us of this. We have now included detailed schematics for each of the custom designed fixtures. The slicing fixture, 1-D transport chamber and non-equilibrium diffusion transport chamber can be found in Figures 2, 4 and 6, respectively. Further Figure 7 now depicts the order of assembly for the non-equilibrium diffusion transport chamber as well as specifications for the plastic inserts and rubber gaskets.

Under Representative Results and Discussion Sections:

The authors frequently refer rather vaguely to the dynamics of binding and "unbinding" as well as carriers binding "too strongly" - are there dissociation constants that have been determined based on previous experiments for optimal, weak, and strong binding that can be used as a reference?

Authors' Response: Weak and reversible binding is essential for solutes to reach the deep zones of cartilage. From the confocal images obtained from the depth of penetration studies, the distances that solutes travel through the matrix of cartilage can be observed. If the solute reaches the deep zone, it is considered to have 'weak' binding; conversely, if a solute remains stuck in the superficial zone it is considered 'strong' binding. These terms are relative to the protein being used based on their charge and composition. For example, Avidin (~7 nm diameter, 66 kDa, pI 10.5) binding with negatively charged matrix glycosaminoglycans (GAGs) has a dissociation constant (K_D) of 150 µM, which is considered to be weak enough to enable the reversible binding necessary for full thickness penetration. Despite the weak binding, Avidin shows high retention and uptake in cartilage due to presence of a high density of negatively charged GAGs (Binding density N_T = 2900 µM) *[Bajpayee et al. Nature Reviews Rheumatology, 2017]*. Further, using thermophoresis, we have recently determined the dissociation constants of CPCs. We noted that CPC +14 had approximately 90-fold stronger binding with cartilage GAGs compared to CPC +8 (K_D = 3 µM vs 272 µM). We intend to present this data in a future publication.

Can the authors provide a size range for what they consider "small" and "large" molecules/carriers?

Authors' Response: Thank you for emphasizing this point. We have now modified the manuscript (lines 362-368) to provide examples of both small and large sized solutes as follows:

"Values >>1 indicate enhanced uptake due to charge interactions, whereas values <1 indicate low uptake. Larger, neutral solutes such as Neutravidin (60 kDa, pI 7) show R_U<1 due to steric hinderance with cartilage matrix *[Bajpayee et al. Nature Reviews Rheumatology, 2017]*, while smaller (neutral solutes are expected to show R_U∼1 as they are able to diffuse into the cartilage reaching equilibrium. In contrast, Avidin (pI 10.5), the positively charged counterpart of Neutravidin, shows an R_U∼180 in cartilage *[Bajpayee et al. Nature Reviews Rheumatology, 2017]*. Further, small-sized CPCs (∼2.5-4 kDa) can show an R_U up to 400 *[Vedadghavami et al. Acta Biomaterialia, 2019]*. As shown by **Figure 9**, the uptake ratios should show a charge-dependent response *[Vedadghavami et al. Acta Biomaterialia, 2019]*."

The authors appropriately acknowledge limitations of the experimental set-up to mimic in vivo conditions and include the possibility of using synovial fluid rather than PBS-PI. The authors should comment on the potential impacts of synovial fluid on transport properties and interactions between the carrier and the cartilage tissue, which can further comprise electrostatic interactions.

Authors' Response: We have now detailed the potential impacts of synovial fluid on the transport properties of charged carriers in cartilage in Lines 584-595 as follows:

"It is estimated that the uptake of cationic carriers in cartilage would decrease in synovial fluid compared to in 1x PBS due to the presence of hyaluronan chains with negatively charged carboxyl groups in synovial fluid. It is possible that cationic carriers competitively bind with the hyaluronan chains of the synovial fluid in addition to the GAGs of cartilage. However, the density of negatively charged groups is significantly higher in cartilage compared to synovial fluid, due to the presence of both negatively charged carboxylated hyaluronan chains and sulfated GAGs in cartilage *[He et al. Journal of Controlled Release, 2020]*. Thus, although the uptake in cartilage in presence of synovial fluid will be lower than in 1x PBS, it is still expected to maintain high intra-cartilage uptake. *In vivo*, Avidin has shown high intra-cartilage uptake in both rat and rabbit cartilage in presence of synovial fluid *[Bajpayee et al. Journal of Orthopaedic Research, 2014; Bajpayee et al. Journal of Orthopaedic Research, 2015]*. Further, Avidin has shown high uptake and retention in cartilage up to 2 weeks following intra-articular injection in a rabbit anterior cruciate ligament transection model *[Bajpayee et al. European Cells and Materials, 2017]*."

Minor Concerns:

In protocol section 1 "Equilibrium Uptake of CPCs in Cartilage"

1.1 Please describe the method used to obtain the cartilage explants. Do the explants need to be obtained from the same region/layer of the cartilage? Should there be an intact superficial layer and/or comprise all the layers of the cartilage?

Authors' Response: Bovine cartilage explants were extracted using dermal punches from the femoropatellar grooves of bovine knee joints supplied from Research 87 (Boylston, MA, USA). We have included a section at the beginning of the protocol to detail this process ("Cartilage Explant Extraction"). Explants extracted from the femoropatellar groove are randomly assigned to experimental groups to prevent bias based on location. The explants are sliced to 1 mm thickness (using the slicing fixture shown in Figure 2) inclusive superficial, middle and deep zones of cartilage. Depending on the species of cartilage, the thickness of the explant may need to be adjusted to obtain multiple zones of cartilage.

1.2 What is the concentration of the protease inhibitor in PBS?

Authors' Response: We have used one mini-tablet in 50 mL of 1x PBS. This information is now included in the protocol for clarity.

1.5 Please specify what is considered "gentle rotation"? Fr example, please provide a reference speed (e.g. 50 RPM with a 0.3cm circular orbit)

Authors' Response: Thank you for highlighting this. We have now added specific rotation requirements in the protocol. Gentle rotation refers to 50 RPM with a 15 mm orbit.

1.6 Should the standard curve be generated at the same time as when you measure your experimental samples ideally? Currently, the protocol reads that they can be done on two different days. Please indicate if timing matters.

Authors' Response: We have clarified this section of the protocol by including a note which reads as follows:

"NOTE: To limit variability in fluorescence readings, incubate the CPC stock solution under the same conditions as the sample plate prior to generation of the standard curve."

In protocol section 2 "Depth of Penetration of CPCs in Cartilage"

2.7 Please specify the rpm used for plate shaker.

Authors' Response: This step of the protocol now has specifications for RPM of rotation.

In protocol section 3 "Non-Equilibrium Diffusion Rate of CPCs in Cartilage"

3.1 typo: "bring" should be "bringing"

Authors' Response: Thank you for noting this error. We have adjusted this step in the protocol to read as follows:

"Bring the two halves of the custom-designed transport chamber **(Fig. 6)** together to assemble and close the chamber. Use washers, nuts and bolts to tightly close the chamber with a wrench."

3.2 s this 0.5% v/v or w/v?

Authors' Response: The concentration of non-fat bovine milk is 0.5% w/v. We have corrected the manuscript for all such instances to avoid confusion.

3.4 In Figure 4, please indicate where the plastic disk and gaskets go, as well as dimensions for the chamber.

Authors' Response: Thank you highlighting this. We have now included Figure 7 which details the dimensions of the plastic inserts and rubber gaskets. The figure also shows the order of assembly for the two halves of the transport chamber. Transport chamber dimensions can now be found in Figure 6.

3.6 Please clarify if the spectrophotometer needs to be modified or any considerations for construction of the chamber in order to obtain these measurements.

Authors' Response: The spectrophotometer used in the non-equilibrium diffusion experiments is a custom designed unit made from translucent PMMA. The CPCs used in these experiments were fluorescently labelled with 5-FAM dye and thus the spectrophotometer laser was designed with a bandpass excitation filter (AT480/30m) with a wavelength of 480 nm in order to excite the fluorescent protein. The receiver was also modified to be equipped with a long pass emission filter (ET515lp) to transmit fluorescent signal emitted from the dye. The laser and receiver brands and wavelengths are now listed in the "Table of Materials" file. We have also added a note to Step 4.7 to detail these requirements. These filters should be chosen in accordance with the excitation and emission of the fluorescent dye used to label the drug carrier.

In the protocol presented, real-time fluorescence measurements are obtained from the custom-designed spectrophotometer which shoots a laser through the translucent transport chamber. However, aliquots from the downstream chamber can be obtained and assessed for fluorescence using a NanoDrop spectrophotometer or plate reader.

Reviewer #2 Comments

Major Concerns:

No major concerns.

Minor Concerns:

Abstract can be improved (more informations about the experiment).

Authors' Response: Thank you for providing your feedback. We have now included further detail about the experimental setups in the abstract as shown below.

"Several negatively charged tissues in the body, like cartilage, present a barrier to targeted drug delivery due to their high density of negatively charged aggrecans and therefore require improved targeting methods to increase therapeutic response. Using the high negative fixed charge density (FCD) of cartilage, drugs can be modified with positively charged drug carriers to take advantage of electrostatic interactions, allowing for enhanced intra-cartilage drug transport. Studying the transport of drug carriers is therefore crucial towards predicting the efficacy of drugs in inducing a biological response. We show the design of three experiments which can quantify the equilibrium uptake, depth of penetration and non-equilibrium diffusion rate of cationic peptide carriers in cartilage explants. Equilibrium uptake experiments provide a measure of solute concentration within cartilage compared to its surrounding bath, which is useful for predicting the potential of a drug carrier in enhancing therapeutic concentration of drugs in cartilage. Depth of penetration studies using confocal microscopy allow for visual representation of 1-D solute diffusion from the superficial to deep zone of cartilage, which is important for assessing whether solutes reach their matrix and cellular target sites. Non-equilibrium diffusion rate studies using a custom-designed transport chamber enables measurement of the strength of binding interactions with the tissue matrix by characterizing the diffusion rates of fluorescently labeled solutes across the tissue; this is beneficial for designing carriers of optimal binding strength with cartilage. Together, the results obtained from the three transport experiments provide a guideline for designing optimally charged drug carriers which take advantage of weak and reversible charge interactions for drug delivery applications. These experimental methods can also be applied for transport evaluation of drugs and drug-drug carrier conjugates. Further, these methods can be adapted for use in targeting other negatively charged tissues such as meniscus, cornea and the vitreous humor."

In the limitations section also please discuss about the limitations of analytical methods and some new references can be added:(computational methods)

J Biomech Eng. 2018 Feb 1;140(2). doi: 10.1115/1.4038716.
Finite Element Framework for Computational Fluid Dynamics in FEBio.
Ateshian GA1, Shim JJ1, Maas SA2, Weiss JA2.

J Biomech. 2012 Apr 5;45(6):1023-7. doi: 10.1016/j.jbiomech.2012.01.003. Epub 2012 Jan 26.
Solute transport across a contact interface in deformable porous media.
Ateshian GA1, Maas S, Weiss JA.

J Vis Exp. 2017 Apr 23;(122). doi: 10.3791/54984.

An Experimental and Finite Element Protocol to Investigate the Transport of Neutral and Charged Solutes across Articular Cartilage.
Arbabi V1, Pouran B2, Zadpoor AA3, Weinans H4.

J Biomech. 2016 Jun 14;49(9):1510-1517. doi: 10.1016/j.jbiomech.2016.03.024. Epub 2016 Mar 21.
Multiphasic modeling of charged solute transport across articular cartilage: Application of multi-zone finite-bath model.
Arbabi V1, Pouran B2, Weinans H3, Zadpoor AA4.

Authors' Response: Thank you for your suggestions. We have added discussion on the limitations of our analytical methods and referenced the aforementioned papers for alternative computational modeling techniques.

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Lead author	Armin Vedadghavami
Title of targeted journal	Journal of Visualized Experiments
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