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Title: Characterization of Intra-Cartilage Transport Properties of Cationic Peptide Carriers

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Author Questionnaire

1. Microscopy: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **N**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y**

If **Yes**, we will need you to record using screen recording software to capture the steps. If you use a Mac, QuickTime X also has the ability to record the steps. Please upload all screen captured video files to your project page as soon as possible.

Videographer: Please film screen capture for steps 3.11. and 3.12. as indicated

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Shikhar Mehta**: Characterizing the transport properties of therapeutics and their carriers is critical for ensuring effective biological responses. These methods aid in designing optimally charged drug carriers for targeting negatively charged tissues **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Shikhar Mehta**: The main advantage of these techniques is their ability to better predict in vivo therapeutic efficacies through a series of in vitro experiments that characterize solute transport through tissue **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

OPTIONAL:

1.3. **Armin Vedadghavami**: Cartilage diseases, such as osteoarthritis, remain untreated due to the inability of drugs to penetrate the dense avascular cartilage matrix, requiring drug carriers to prolong therapeutic efficacy **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Protocol

2. Cartilage Cationic Peptide Carrier (CPC) Equilibrium Uptake

2.1. Begin by using a delicate task wipe to gently remove excess PBS from the surfaces of 3-millimeter-diameter, 1-millimeter-thick cartilage explants **[1]**.

2.1.1. WIDE: Talent dabbing explant(s)

2.2. Use a balance to quickly record the wet weight of each explant **[1]** and immediately place the explants into a PBS bath to prevent dehydration **[2]**.

2.2.1. Talent placing explant onto balance

2.2.2. Talent placing explant into bath, with PBS container visible in frame

2.3. Next, add 300 microliters of freshly prepared 30-micromolar, fluorescently labeled cationic peptide carrier solution per well to the inner wells of a 96-well plate **[1]** and use a spatula to add one explant to each well of solution **[2]**.

2.3.1. Talent adding solution to well(s)

2.3.2. Talent adding explant to well(s)

2.4. Fill each of the surrounding wells with 300 microliters of PBS **[1]** and cover the plate with a lid **[2]**.

2.4.1. Talent adding PBS to well(s)

2.4.2. Talent placing lid onto plate

2.5. Seal the edges of the plate with flexible film to minimize evaporation **[1]** and place the plate on a shaker in a 37-degree Celsius incubator for 24 hours at 50 revolutions per minute with a 15-millimeter orbit **[2]**.

2.5.1. Talent sealing edges

2.5.2. Talent placing plate onto shaker

2.6. At the end of the incubation, transfer the equilibrium bath from each well into individual polypropylene tubes **[1]** and make serial dilutions from the stock 30-micromolar cationic peptide carrier solution to generate a standard curve **[2]**.

2.6.1. Talent adding bath to tube(s)

2.6.2. Talent serially diluting stock solutions in tubes, with stock solution and dilution labels visible in frame NOTE: This is slated as 2.6.1b

2.7. Then transfer 200 microliters of each solution and standard into individual wells of a black 96-well plate **[1]** and obtain fluorescence readings of each sample and standard based on the excitation and emission wavelengths of the fluorescent label **[1-TXT]**.

2.7.1. Talent adding solution to well(s)

2.7.2. Talent loading plate onto plate reader **TEXT: Dilute samples in PBS-PI as necessary**

3. CPC Cartilage Depth Penetration Analysis

3.1. To determine the depth of cationic peptide carrier penetration within a cartilage explant, use a scalpel to cut 6-millimeter-diameter, 1-millimeter-thick cartilage explants in half **[1]** and hydrate the resulting half-disk pieces with protease inhibitor-supplemented PBS **[2]**.

3.1.1. WIDE: Talent cutting explant(s)

3.1.2. Talent adding PBS-PI to tissue(s), with PBS and inhibitor container visible in frame

3.2. Apply epoxy to the center of a well of a custom-designed 1-dimensional transport chamber **[1]** and secure one half-disk explant within the well with the superficial side of the explant facing the upstream side of the chamber **[2]**.

3.2.1. Talent applying epoxy to well *Videographer: Important step*

3.2.2. Explant being placed into well *Videographer: Important step; Video Editor: please emphasize superficial side*

3.3. Remove any excess glue from the well to prevent contact with the diffusion surface area of the cartilage **[1]** and add 80 microliters of protease inhibitor-supplemented PBS to both sides of the explant **[2]**.

3.3.1. Glue being removed

3.3.2. PBS-PI being added to well, with PBS-PI container visible in frame

3.4. Pipette the liquid up and down on one side of the explant to check for leakage to the other side **[1-TXT]**.

3.4.1. Liquid being pipetted up and down **TEXT: Readjust explant and apply epoxy as necessary**

3.5. If no leakage is present, replace the protease inhibitor-supplemented PBS from the upstream side with 80 microliters of 30-micromolar, fluorescence-labeled cationic peptide carrier solution **[1]** and carefully place the transport chamber into a cell culture dish **[2]**.

3.5.1. CPC solution being added to well

3.5.2. Talent placing chamber into dish

3.6. Cover the base of the dish with PBS to avoid cationic peptide carrier solution evaporation **[1]**, taking care that there is no direct contact between the solutions from the upstream and downstream chambers **[2]**.

3.6.1. PBS being added to dish

3.6.2. Shot of lack of contact between solutions *Video Editor: please emphasize up- and downstream chambers when mentioned*

3.7. Place the covered dish on a shaker to limit particle sedimentation for 4 or 24 hours at room temperature and 50 revolutions per minute with a 15-millimeter orbit **[1]**.

3.7.1. Shot of dish on shaker

3.8. At the end of the incubation, remove the explants from the chamber **[1]** and cut approximately 100-micron-thick slices from the center of each explant **[2]**.

3.8.1. Explant being removed *Videographer: Important/difficult step*

3.8.2. Explant being cut *Videographer: Important/difficult step*

3.9. Place each slice of explant between a glass slide and a coverslip **[1]** and hydrate the slices with fresh protease inhibitor-supplemented PBS **[2]**.

3.9.1. Coverslip being placed onto slice on slide *Videographer: Important step*

3.9.2. PBS-PI being added to slice *Videographer: Important step*

3.10. Fix the slide on to the stage of a confocal microscope **[1]** and obtain a z-stack of fluorescent images through the full thickness of the slice at 10x magnification **[2]**.

3.10.1. Talent loading sample on microscope stage, imaging slice

3.10.2. Talent opening image(s), with monitor visible in frame

3.11. Open the image file in ImageJ, click **Image** and select **Stacks** and **Z Project** from the dropdown menu **[1]**. NOTE: 3.11.1. and 3.12.1. are uploaded as 1 video file.

3.11.1. SCREEN: To be provided by Authors: Image, Stacks, and Z project being selected

3.12. Then input the slice numbers from 1 to the final slice and, under **Projection Type**, select **Average Intensity** and click **OK [1]**.

3.12.1. SCREEN: To be provided by Authors: Slice numbers being entered, then Average Intensity being selected and OK being clicked

4. Non-Equilibrium CPC Cartilage Diffusion Rate

4.1. To assess the non-equilibrium cationic peptide carrier cartilage diffusion rate, assemble each half of the transport chamber to include 1 large rubber gasket **[1]**, 1 polymethylmethacrylate insert **[2]**, and 1 small rubber gasket **[3]**.

4.1.1. WIDE: Talent placing gasket into chamber *Videographer: Important step*

4.1.2. Talent placing insert into chamber *Videographer: Important/difficult step*

4.1.3. Talent placing gasket into chamber *Videographer: Important/difficult step*

4.2. Place the explant in the wells of the plastic insert with the superficial surface facing the upstream chamber **[1]** and sandwich the two halves together to complete the assembly **[2]**.

4.2.1. Talent placing explant into well *Videographer: Important step*

4.2.2. Halves being sandwiched *Videographer: Important step*

4.3. Use a wrench to tightly screw the halves together **[1]** before filling the upstream chamber with 2 milliliters of protease inhibitor-supplemented PBS **[2]**.

4.3.1. Sandwich being tightened

4.3.2. Chamber being filled

4.4. Check the downstream chamber for leakage from the upstream chamber **[1-TXT]**. If no leakage is detected, fill the downstream chamber with 2 milliliters of protease inhibitor-supplemented PBS **[2]**.

4.4.1. Shot of transport chamber **TEXT: Reassemble chamber, adjust gasket position, and tighten screws as necessary**

4.4.2. Downstream chamber being filled

4.5. Add a single mini-stir bar to both the up- and downstream chambers **[1]** and place the chamber on a stir plate **[2]** with the chamber aligned such that the laser from the spectrophotometer is focused toward the center of the downstream chamber **[3]**.

4.5.1. Stir bar being added *Videographer: Important step*

4.5.2. Talent placing chamber onto plate NOTE: 4.5.2 and 4.5.3 combined

4.5.3. Chamber being aligned *Videographer: Important step*

4.6. With the signal receiver portion of the spectrophotometer behind the downstream chamber **[1]**, collect stable real-time downstream fluorescence emission readings for at least 5 minutes **[2]**.

4.6.1. Shot of receiver behind transport chamber

4.6.2. SCREEN: 4.6.2 5 min stable signal: Author NOTE: Please show 00:10-01:10 (60 seconds sped up and shown in 10 seconds) *Video Editor: please emphasize Fluorescence window/data*

4.7. After obtaining a stable reading, add a pre-calculated volume of fluorescently-labeled cationic peptide carrier stock solution to the upstream chamber to a final bath concentration of 3-micromolar **[1]** and monitor the downstream fluorescence signal while allowing the solute transport to reach a steady increase in slope **[2-TXT]**.

4.7.1. Talent adding solution to upstream chamber

4.7.2. SCREEN: 4.7.2 Shot of downstream: Author NOTE: Please show 00:00-01:30, 90 sec sped up and shown in 10 seconds *Video Editor: please emphasize Fluorescence window/data*

4.7.3. SCREEN: 4.7.3 Reaching steady state slope: Author NOTE: Please show 00:00-03:00 (3 min sped up and shown in 10 seconds) *Video Editor: please emphasize Fluorescence window/data* **TEXT: Thicker explants require longer steady state acquisition**

4.8. Once a steady state has been reached, transfer 20 microliters of solution from the upstream chamber to the downstream chamber for a spike test **[1]** and collect the real-time downstream fluorescence readings **[2]**.

4.8.1. Solution being transferred

4.8.2. SCREEN: 4.8.2 Spike test: Author NOTE: 00:03-11:00 *Video Editor: please speed up* NOTE: I would just show the final readings here.

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?

3.3., 3.8., 3.9., 4.1., 4.2., 4.5.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?

3.8. Cutting and obtaining 100-200 micrometer thick cartilage slices is one of the most difficult steps in the protocol. In order to obtain thin cartilage slices of uniform thickness, practice is required. Further, use of a new, sharp and straight edged blade is ideal for cutting.

4.1. Assembly of the transport chamber components in a leak-proof manner can be difficult. Gasket and explant thickness and placement are critical to ensuring complete closure of the transport chamber. Further, tightening of screws using a wrench is necessary.

Results

5. Results: Representative Cartilage Depth of Penetration and Non-Equilibrium Diffusion Analyses

5.1. Too high a positive charge will limit solute penetration to the superficial zone, as the carrier binds too strongly to the matrix **[1]**.

5.1.1. LAB MEDIA: Figure 10 *Video Editor: please emphasize +20 images*

5.2. Conversely, carriers that are able to take advantage of weak and reversible charge interactions penetrate through the deep zone of tissue **[1]**.

5.2.1. LAB MEDIA: Figure 10 *Video Editor: please emphasize +8 and +14 images*

5.3. An optimally charged drug carrier, however, would not only penetrate the deep zones of tissue **[1]** but would also demonstrate a high intra-tissue uptake **[2]**.

5.3.1. LAB MEDIA: Figure 10 *Video Editor: please outline 24 hour +14 images*
5.3.2. LAB MEDIA: Figure 9 *Video Editor: please outline +14 bar*

5.4. Non-equilibrium diffusion transport experiments result in a data-generated curve with a gradually increasing slope **[1]**.

5.4.1. LAB MEDIA: Figure 11

5.5. The initial part of the curve represents solute diffusion through the cartilage as solute-matrix binding interactions occur **[1]**.

5.5.1. LAB MEDIA: Figure 11 *Video Editor: please emphasize curve from 0-40*

5.6. Once solutes reach the downstream chamber **[1]**, the slope of the curve increases as the fluorescence readings increase over time **[2]**.

5.6.1. LAB MEDIA: Figure 11
5.6.2. LAB MEDIA: Figure 11 *Video Editor: please emphasize data line from 40-end of red triangle*

5.7. This second part of the curve then reaches a steady slope, representing steady state diffusion **[1]**.

5.7.1. LAB MEDIA: Figure 11 *Video Editor: please add D_{SS} Slope text*

5.8. The x-intercept of a tangential line drawn at the steady state portion of the curve indicates the time it takes to reach steady state diffusion, or Tau-lag **[1]**.

5.8.1. LAB MEDIA: Figure 11 *Video Editor: please add Tau Lag text and arrow*

5.9. Following solution transfer from the upstream to the downstream chamber, a spike in fluorescence is observed **[1]**, at which point the stabilized fluorescence intensity can be used to correlate the fluorescence to the concentration **[2]**.

5.9.1. LAB MEDIA: Figure 11 *Video Editor: please emphasize data line spike at end of red triangle to end of graph*

5.9.2. LAB MEDIA: Figure 11

5.10. The representative effective diffusivity **[1]** and steady state diffusion values can then be calculated as indicated **[2-TXT]**.

5.10.1. LAB MEDIA: Table 1 *Video Editor: please emphasize D_{EFF} and D_{SS} data column*

5.10.2. BLACK TEXT ON WHITE BACKGROUND: $\frac{\partial}{\partial t}\left(\frac{C_D}{C_U}\right) \cong \frac{\Phi D_{ss} A}{L V_D}$

Conclusion

6. Conclusion Interview Statements

6.1. **Shikhar Mehta**: Maintain explant hydration and minimize solution evaporation throughout the experiment to prevent changes in the cartilage morphology and the solution concentration, respectively, and to ensure accurate and reproducible data acquisition **[1]**.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

6.2. **Armin Vedadghavami**: Following the design of an optimally charged drug carrier, various conjugation techniques can be utilized to modify drugs for enhanced tissue targeting and to facilitate evaluation of their biological efficacy **[1]**.

6.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera