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**JoVE Science Education Series: Biomedical Engineering**

**Title: Biodistribution of Nano-Drug Carriers: Applications of SEM**

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Notes to authors are highlighted

1. **Overview** 
   1. Metallic and magnetic nanoparticles are widely being used as nano-carriers for drug delivery and their biodistribution in tissues is essential to evaluate their therapeutic efficacy and safety.
      1. Title Slide.
   2. Nanocarriers are sub-micron particles, usually limited to less than 200 nm, that can be loaded with therapeutic agents (1.2.1). Because of their size, they are able to access many sites and organs in the body (1.2.2). Where the particles end up in the body, called their biodistribution is an important parameter used to evaluate safety, optimize dosing and improve drug targeting. (1.2.3).
      1. See storyboard
   3. In this video, the basic principles of targeted drug delivery will be described (1.3.1), and a method to evaluate biodistribution using high resolution imaging techniques will be demonstrated (1.3.2). Other applications of nanoparticle-based carriers will also be discussed (1.3.3).
      1. Reuse 2.4.3 (Passive vs active targeting)
      2. Use shot: 3.4.2. (SEM image of nanoparticles)
      3. Use shot: 5.2.4 (10261@ 09:26-10:00: Vaccine being administered)
2. **Principles of Nanocarrier Drug Delivery**
   1. Let us begin by discussing the fundamentals of nanoparticles and understand why they are being developed as drug carriers.
      1. Title slide
   2. First, nano-scale particles, which can be either polymeric, liposomal, or metallic, are usually biocompatible--meaning they are not harmful or reactive to living tissue and do not elicit an immune response (2.2.1). However, nano-toxicology studies must be performed in order to understand how the materials and particle size affect biodistribution in the body. (2.2.2)
      1. See storyboard

* 1. Second, their small size allows their extravasation through the endothelium at inflammatory sites, such as in tumors, and results in efficient cellular uptake (2.3.1).
     1. See storyboard
  2. As cancer cells divide, a vascular supply is needed to supply nutrients and oxygen and support tumor growth (2.4.1). These blood vessels form rapidly and therefore are usually abnormal and defective, containing large gaps in their endothelial lining resulting in a leaky vasculature and an increase in permeability (2.4.2).
     1. See storyboard.
  3. The nanoparticles are then able to escape from the bloodstream and accumulate within the tumor microenvironment (2.5.1).
     1. See storyboard.
  4. This is called passive targeting, where the nanocarrier reaches the target organ through a phenomenon known as the EPR effect, or the enhanced permeability and retention effect (2.6.1).
     1. See storyboard.
  5. Finally, these nanoparticles have a large surface area that can be functionalized with specific ligands such as antibodies, or proteins (2.7.1).
     1. See storyboard
  6. In active targeting, these ligands then recognize and bind to receptors that are overexpressed by cells in the tumor site (2.8.1).
     1. See storyboard
  7. Specific interactions between the ligands on the nanocarrier surface and the cell receptors triggers receptor-mediated endocytosis and facilitates cellular uptake (2.9.1).
     1. See storyboard
  8. Now that we understand the basics of nanoparticle drug delivery (2.10.1), let’s see a demonstration that uses high resolution imaging (2.10.2.) to determine the biodistribution of metallic nanoparticles in a mouse model (2.10.3).
     1. Reuse 2.5.2 (passive targeting)
     2. Reuse 3.2.1 (talent loading sample)
     3. Reuse 3.5.3 (Talent imaging mouse tissue at workstation)

1. **Nanoparticle Injection and Organ harvesting**
   1. To begin the biodistribution study, first, inject an anesthetized mouse with nanoparticles, and allow the nanoparticles to passively target the organ over time. For this study, 30 nm barium and titanium particles were injected intravenously (3.1.1).
      1. Archival footage
   2. At 1, 4, and 8 weeks post-injection, humanely euthanize the mice according to AVMA Guidelines. Then, open the body cavity, and surgically remove the organs of interest for further analysis. Harvest the spleen, kidney, liver and lungs, and store the organs in 10% phosphate buffered formalin in a polypropylene container until sample preparation(See Sci Ed Jove Video: [Basic Biology](https://www.jove.com/science-education/basicbio)> [Lab Animal Research](https://www.jove.com/science-education-library/23/lab-animal-research): Diagnostic Necropsy and Tissue Harvest)
      1. Archival footage
2. **Tissue Sample Preparation**
   1. Now, use forceps to transfer the mouse tissue from the fixative into phosphate-buffered saline (4.1.1). Rock the sample for 30 minutes (4.1.2), replacing the PBS every ten minutes to remove excess fixative (4.1.3).
      1. **MED-over the shoulder**: Talent uses forceps to transfer the mouse tissue from the fixative into the PBS.
      2. **MED**: talent places the sample in PBS on a rocker/shaker and sets timer for 30 minutes
      3. **MED**: talent removes the PBS using a pipette, and adds new PBS.
   2. Then, remove the tissue from the shaker (4.2.1). Add optimal cutting temperature compound, which contains water soluble glycols and resins, to a labeled plastic mold (4.2.1.B). Dry the tissue with a kimwipe (4.2.2) before placing it in the plastic mold. Fill the mold with OTC compound, covering the tissue, and place in a plastic bag (4.2.3). Set the bag in a bucket containing dry ice (4.2.3B), and move to a -80C freezer overnight (4.2.4).
      1. **MED**: talent stops the rocker and removes the sample from the rocker

4.2.1b : Talent adds compound to a plastic mold

* + 1. **CU**: talent removes the sample from PBS solution and dries with a kimwipe.
    2. **MED/CU**: talent places the tissue in plastic mold, covers with more OCT and stores in a plastic bag

4.2.3b: Talent places sample on dry ice

* + 1. **WIDE/MED**: talent transports the mold to the -80 freezer, opens the door and places the sample inside, and shuts the door.
  1. The next day, remove the sample from the freezer, and place on dry ice while transporting to the cryostat (4.3.1). Set the chamber temperature to -23° (4.3.2A), and then transfer the sample to the cryostat (4.3.2b). Label slides with the organ type and nanoparticle size of the sample you will be sectioning (4.3.3)~~, and place them on a shelf in the cryostat.~~ Then, activate the cryostat (4.3.4).
     1. **WIDE/MED**: Talent opens the -80, removes the sample and carries to the cryostat

**4.3.2A MED-over the shoulder**: Talent sets the temperature to -23. Please show the inside of the cryostat.

4.3.2B Talent opens the cryostat and places the sample in the chamber

**4.3.3MED/CU**: Talent is at a nearby bench, and labels the slide with organ type and nanoparticle size. Please show at least two slides being labeled.

**4.3.4MED-over the shoulder**: Talent presses cryobar boost to activate the cryostat

*Please make sure to indicate the organ type and nanoparticle size so we can incorporate into the voiceover.*

* 1. Next, cover the cryostat chuck with O.C.T (4.4.1). Then remove the sample from the mold, and place it on top of the chuck. The sample is frozen (4.4.2). Mount the chuck onto the specimen holder and orient/ and adjust so the blade will cut straight across the frozen sample (4.4.3).
     1. **CU**: Talent adds OCT to the chuck
     2. **MED/CU**: Talent removes the sample from the mold and gently puts it on top.
     3. **MED**: Talent detaches the chuck and moves to the specimen holder. Talent tightens and adjusts to make sure the sample and blade are properly aligned.
  2. Now, bring the sample closer to the blade, and set the thickness to 30 um for rough facing (4.5.1). Rotate the hand wheel to slice 30 um-thick sections (4.5.2), and continue sectioning until an even tissue slice is cut (4.5.3).
     1. **CU**: Talent moves the specimen closer to the blade, and turns the dial to set the thickness to 30um.
     2. **MED/MED-over the shoulder**: Talent rotates the hand wheel (exterior of cryostat)
     3. **CU**: Slices of tissue being sliced. Show initial slices, and several more slices up until when the sample is ready for fine facing.
  3. For fine facing, decrease the section thickness to 7-8 um. (4.6.1). Collect the sections by pressing a labeled glass slide on the slice. (4.6.2). Then, add the slides to the rack, and air dry at room temperature (4.6.3).
     1. **MED**: Talent switches the thickness to 7-8 um, and slices a section.
     2. **CU**: Talent presses a glass slide onto the freshly sliced section. Then talent slices another section and presses the same glass slide onto a second sliced section. Please make sure to capture at least two slices being sectioned and then stuck to slide.
     3. **MED-over the shoulder**: Talent adds the slide to the rack (the rack should already contain some slides)
  4. Once dry, gently dip the slide rack in 50% ethanol for 3 minutes to remove the OCT (4.7.1). Then, transfer the rack to 80% ethanol for 3 minutes (4.7.2), before placing the rack in a 1:1 ratio of cold methanol to acetone (4.7.3A) for 10 minutes at -20C (4.7.3B).
     1. **MED/MED-over the shoulder**: Talent repeatedly dips the slide rack in 50% ethanol. Label of 50% ethanol should be clearly visible.
     2. **MED/MED-over the shoulder**: Talent removes the slide rack from 50% ethanol and transfer to 80% ethanol

**4.7.3AMED/MED-over the shoulder**: Talent transfers the rack to cold methanol/acetone

**4**.7.3B Talent places in the freezer.

* 1. Then, remove the slide rack, and drain on a paper towel. (4.8.1). After 20-30 minutes, place the slides in a slide box, and store in a freezer at -20C until use (4.8.2).
     1. **MED**: Talent removes the slide rack and places on a paper towel to drain off the solution.

**4.8.2A MED**: Talent removes the slides from the slide rack, places in a slide box

**4**.8.2B: Talent then walks slide box to freezer. Talent places slide box in the freezer.

1. **High Resolution Imaging using SEM and EDS**
   1. Now, let’s image mouse lung tissue that was collected 1 week post-injection with 30 nm barium and titanium particles to determine their biodistribution.
      1. Title slide
   2. To begin (5.2.1), first mount a prepared slide onto the SEM stage (5.2.2). Watch JoVe’s Video on "Biological Sample Preparation and Basic Image Acquisition for SEM" to learn how to sputter coat your sample (**TEXT: Instructions in "Biological Sample Preparation and Basic Image Acquisition for SEM**"). Then, load the stage into the SEM chamber. (5.2.3).
      1. **WIDE**: Establishing shot of talent approaching SEM carrying the sample and SEM stage.
      2. **MED-over the shoulder**: Talent mounts a sputter-coated slide on the SEM stage, and tightens the screw.
      3. **MED**: Talent opens the chamber, places mounted slide inside and closes it.
   3. Once the sample is in the field of view, move the sample vertically to a working distance of approximately 5 mm (5.3.1).
      1. **MED-Over the shoulder/SCREEN**: Talent moves the stage using the controller until the sample is in the field of view. We should see the sample moving on the computer screen as the talent adjusts the working distance.
   4. Turn the electron beam on and select the detector for secondary electrons. **(TEXT: SE2)**. Then, set the beam accelerating voltage to 25 keV (5.4.1).
      1. **SCREEN**: Talent turns on the electron beam and then selects SE2 from the detector options before adjusting the beam accelerating voltage to 30 keV through the software
   5. To begin imaging, zoom in on the sample to a magnification of approximately 1,000-2000 x. At this magnification, the structure that contains the nanoparticles should be visible, even though the nanoparticles are not. This is called the secondary image (5.5.1) (**TEXT: Secondary image**).
      1. **SCREEN**: Talent looking at SEM workstation monitor and zooming in on the sample. Show image of sample at high magnification, and that the magnification is ~1000-2000x.
   6. Now, engage the back-scattered electron detection mode in the SEM module to visualize the nanoparticles. Move the stage in the z-direction to attain the same 5 mm working distance used above (5.6.1).
      1. MED-over the shoulder/**SCREEN**: Talent selects backscatter mode and then adjusts the z-position of the stage using the joystick (The BSE is already in the system and will be placed using the computer. The screen will show the insertion of the detector through the in-chamber camera).
   7. Adjust the configuration of the BSD and use different voltage biases for the detection panels until the image is crisp (5.7.2) Regions of high contrast, the nanoparticles, should now be visible. This is the backscattered image. Capture and save the image (5.7.1).
      1. **SCREEN**: High resolution image of sample in back scatter detection mode. Talent captures and saves the image.
      2. **SCREEN**: Talent adjusting the configuration of the BSD using the software—make sure to show adjustments and final crisp image.
   8. Next, attain energy dispersive X-ray spectroscopy, or EDS, data of the sample. Zoom in on the high contrast area of a clump of nanoparticles. Then, open the 2nd camera in the chamber, and lower the EDS into the system. Watch the camera screen to ensure that the EDS approaches, but does not touch the BSD or the electron gun (3.7.1).
      1. **MED-over the shoulder/SCREEN**: Talent zooming in on high contrast area and then opening the 2nd camera on the SEM software and lowering EDS into position. Continue capturing until EDS is in position.
   9. Then, open the microanalysis software and acquire an image. Use the mouse to select a region of interest for further analysis (3.8.1).
      1. **MED-over the shoulder/SCREEN:** Talent opening Aztec program and capturing an image. Then talent selects/clicks a region of high contrast.
   10. An X-ray spectrum for that area is then displayed (3.9.1). Here, the peaks represent barium and titanium, confirming the presence of metallic nanoparticles in the sample (3.9.2).
       1. **SCREEN:** X-ray spectrum for that area, and then overlaid with barium and titanium at the respective peaks.
   11. Now, open qualitative data analysis software and map the borders of the organ on the slide. Then, select and run the appropriate protocol from the menu (3.10.1) to create a mosaic image of the organ. This may take several hours (3.10.2).
       1. **SCREEN***:* Talent opens the Atlas software and mapping the borders of the organ. Then talent selects and runs the "organ" protocol from the menu. Show the screen as the program begins to stitch the image together. Try to show some progress in images being stitched together.
       2. **SCREEN**: Final mosaic image
   12. Once complete, export it as a Tif file, and open the file in ImageJ (3.11.1). Adjust the contrast threshold values to highlight areas of very high contrast--the nanoparticles (3.11.1). 
       1. **MED-over the shoulder/SCREEN**: Talent exporting the final image as a tif file and opening in imageJ. Then talent adjusting the threshold. *Make sure to show changes in the contrast and very high contrast.*
   13. Then, use built-in functions to define the area of the organ and the pixel size threshold (**TEXT: ~100 nm)**. Select “Analyze Particles” to obtain the average number of pixels per grouping and the percentage of the organ covered with nanoparticles (5.13.1).
       1. **SCREEN**: talent using built-in functions to define the area and adjusting pixel size threshold. performing the function that will quantify the number of nanoparticles. Then talent selecting analyze particles and showing the final result
   14. Repeat all steps in this procedure for remaining tissue samples from other time points and organs (3.13.1). Once all data is collected, compile it into a biodistribution graph (3.13.2).
       1. Reuse 5.2.3 and then 5.9.1 (Talent loading sample, then selecting region of high contrast)
       2. **WIDE/MED**: Talent at computer workstation making a biodistibution graph.
2. **Results**
   1. Now, let’s analyze the images to determine the biodistribution and learn how the body processed the nanoparticles (4.1.1).
      1. *Title slide*
   2. First, plot the measured particle distribution as a function of time for all samples analyzed. This is the distribution of 30 nm-sized nanoparticles in various mouse organs over time (4.2.1).
      1. See storyboard
   3. There is an overall decrease in nanoparticles after 8 weeks, which indicates clearance of the nanoparticle from the body (4.3.1). However, there is increased nanoparticle concentration in the liver after 4 weeks. This suggests that the body may be processing the 30 nm barium and titanium nanoparticles used in this study as a toxin. (4.3.2)
      1. See storyboard
   4. This analysis can also be performed to evaluate how the size of the nanoparticle affects its biodistribution in the body. Changing the size of the nanoparticles affected the rate of overall cellular uptake and the rate of clearance (4.4.1).
      1. See storyboard.
3. **Applications**
   1. Nanoparticles and nano-carriers are widely used in biomedical research and have applications as imaging, diagnostic, and therapeutic agents.
      1. Title slide.

* 1. **(Lower third: Application #1- vaccine delivery)** Nanoparticles are being developed for use in vaccine delivery (5.2.1) against a wide variety of infectious diseases (5.2.2) because they protect vaccine components from degradation (5.2.3) and maximize immune stimulation (5.2.4).
     1. JoVE 53102 @ 1:48-1:56 (wide shot of talent working at bench, then closer up shot of scooping powder into glass vial)
     2. JoVE 53102 @ 2:17-2:20 (talent adding solution to vial)
     3. JoVE 10261 @ 5:53-5:58 (nurse filling syringe)
     4. JoVE 10261 @ 9:26-10:00 (show ‘vaccine’ being administered and needle being removed from skin)
  2. Interbilayer-crosslinked multilamellar vesicles, or ICMVs (5.3.1) are being developed (5.3.2) for the induction of antigen-specific CD8+ T cell responses (5.3.3). These ICMVs (5.3.4) specifically localize in the lymph nodes of mice for efficient vaccine delivery (5.3.5) and have elicited robust immune responses against malarial antigens and tumor cells (5.3.6).
     1. JoVE 52771 @ 2:02-2:06 (talent pipetting into glass vial labeled ICMV)
     2. JoVE 52771 @ 2:34-2:36 (talent placing glass vial on vortexer)
     3. JoVE 52771 @ 2:40-2:45 (talent pipetting into tube—please show wide view and then medium view)
     4. JoVE 52771 @ 3:44 (close up of tube with white clump of nanoparticles at the bottom)
     5. JoVE 52771 @ 6:40-6:44 (Talent at workstation with mice on computer screen)
     6. JoVE 52771@ 9:27-9:36 (Graph with 4 panels, then the bottom right panel is enlarged showing the ‘red’ lymph nodes)
  3. **(Lower third: Application #2-Imaging)** Metallic nanoparticles are often used as contrast agents in magnetic resonance imaging (5.4.1) to visualize tissue structure and function for early disease detection (5.4.2).
     1. 57128 @ 1:43-1:50 (patient laying outside of MRI)
     2. 57128 @9:00-9:10 (full body MRI scan)
  4. Iron oxide nanoparticles are useful diagnostic probes (5.5.1). When synthesized with a bisphosphonate moiety (5.5.2), these nanoparticles quickly and selectively accumulate in atherosclerotic plaques and allow their visualization within 1 hr for a rapid diagnosis (5.5.3).
     1. JoVE 53472 @1:48-1:56
     2. Jove 53472 @3:18-3:20
     3. JoVE 53472 @ 6:51
  5. Recently, loaded nanocarriers have been developed as a strategy to simultaneously detect early stage cancer and deliver chemotherapeutic agents. These nanocarriers are called theranostics because they integrate diagnostic and therapeutic abilities (5.6.1).
     1. See storyboard

1. **Summary**

You’ve just watched JoVE’s video on determining the biodistribution of nano-drug carriers (6.1.1). You should now know the basic principles of nanodrug carriers (6.1.2), how to detect nanocarriers in tissue samples using high resolution SEM (6.1.3) and determine their biodistribution (6.1.4), and some applications of nanoparticles in biomedical engineering (6.1.5). Thanks for watching!

* + 1. Title slide
    2. Reuse 2.5.2
    3. Reuse 3.5.4 (talent adjusting parameters for backscatter electron detection)
    4. Reuse 3.8.3 (Mosaic image showing the nanoparticles in the mouse sample)