Appendix A

Content

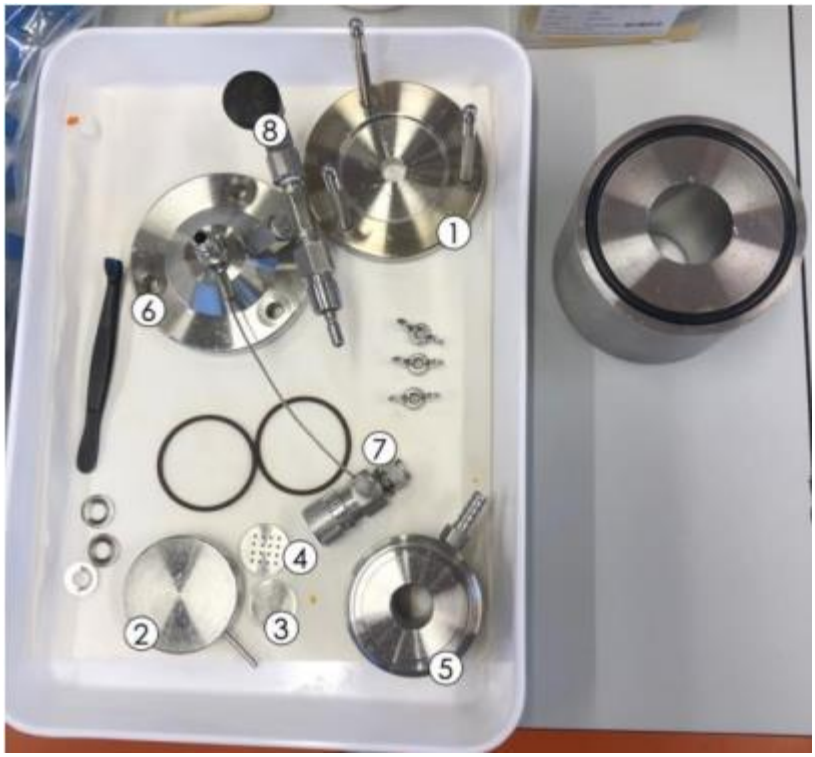
Thin-film hydration

Make sure someone has shown how to perform these steps before you do!

1. Take the lipids DPPC, DSPE-PEG2000 and cholesterol (+DiD-label if you want to label your liposomes)(+DSPE-PEG2000-Biotin for release test) out of the freezer (3.38 V1). It will take about 30 minutes before the lipids are ready to use.
2. Switch on the Cryostat (cooler) and put the water bath at 65°C of the Rotavapor R210. Make sure than a thick layer of water is present in the bath.
3. Prepare hydration buffer → 50 mL of hydration buffer (Ammonium Sulfate 300 mM, pH 4). (See Appendix B, Hydration buffer).
4. Grab a beaker and a round flask, place tissues in the beaker and the round flask on the tissues in the beaker. Label the flask accordingly (Type of liposomes, date, name, etc).
5. After the 30 minutes are done, weigh out the lipids. For amount/concentrations of lipids (see Appendix B, lipid mixture for liposome preparation).
6. Dissolve Lipids in 1 mL Chloroform + Methanol (2:1)(does not have to be precise as it will be evaporated later) in the fume hood. Make sure that leftover lipids on the neck of the flask are rinsed to the bottom of the flask when adding the chloroform and methanol.
7. Attach the flask to the rotavapor in the other fume hood with 2 orange/red clips and the fitting glass "connecter". Start the aspirator (on the far left) and turn on the vacuum by turning the glass valve towards you. Make sure that the round flask is in contact with the water. Use maximum rotation and 65 degrees Celsius water bath.
    a. Note: PEG can start a foam process during the evaporation process. Make sure the foam does not reach the neck of the rotavapor by pointing the pressure valve downwards lower the pressure. This will remove the foam.
8. Start the rotavapor, 200 mbar, 65°C, maximum rotation for 30 minutes.
9. Make sure all the lipids are dried out on the surface of the flask. You can check this by heightening the rotavapor and stop the rotation once in a while. When DiD-label is added, this step will take a bit longer as more liquid is present. When the lipids are dried, the thin-film layers are made.
10. When all the lipids are dried out, stop the rotavapor by pressing the red button on the operating panel, turn off the aspirator and aim the pressure-valve downwards to release the pressure and vacuum. Remove the flask. Do not turn the warm bath and cooler of as you will be using it again after 30 minutes.
    a. Note, do **not** remove the flask before the pressure is back to normal (just below 1000 mbar), or the pressure will pull thin layer from the flask in the Rotavapor.
11. Flush the flask with N2. Place the end of the N2 tube in the flask. Under the fume hood, on the right: turn the right lever all the way down and the left a quarter. This activates the N2 release, you should hear this is well.
12. When the N2 flush is done, add X mL of hydration buffer (if you want X mL of liposomes) to the round flask.
13. Place flask in the rotavapor at maximum rotation and 65 degrees Celsius to dissolve the lipids in the buffer. The hydration that occurs during this step, leads to the production of liposomes. If no DiD-label is used, the liquid will turn white from the liposomes. If DiD-label is used, it will be blue. Remove flask from water bath occasionally to see if the thin-film layer has dissolved.
14. Stop the rotavapor: press the red button on the operating panel Stop rotating the flask. Remove the flask and put a cap on it. Turn off the warm bath and cooler.
15. Place the liposomes in a 15 mL tube, label it, and store in the fridge until the next step.

Extrusion

Make sure someone has shown how to perform these steps before you do!



1. Take the tube with liposomes from the fridge. Take the tube and the hydration buffer (see Appendix B, Hydration buffer) and take it to the extruder. Take 8 15 mL tubes.
2. Assemble the Extruder as followed: (A figure is given above for the explanation).
3. Place the filter support base (2) onto the tie rod base (1). Place disc 3 in 2. Place disk 4 on 3.
4. Place the Whatman Nucleopore Track-etched membranes (Sigma), the white thin ones, on disk 4 in the following order
    a. Combination 1, 400 nm + 600 nm (400 on 4, 600 on 400). Extrude 2 times
    b. Combination 2, 200 nm + 400 nm (200 on 4, 400 on 200). Extrude 5 times
    c. Combination 3, 200 nm + 200 nm. Extrude 10 times
    d. Combination 4, 100 nm + 200 nm (100 on 4, 200 on 100). Extrude 20 times
    e. Combination 5, 100 nm + 100 nm. Extrude 15 times
    120 nm liposomes are done now
    f. Combination 6, 50 nm + 100 nm (50 on 4, 100 on 50). Extrude 20 times
5. Place the Black O-ring on the nucleopores.
6. Place 5 on 2. Make sure that the rubber bands are on the opposite sites, not touching each other. Place the top part (6) on part 5 and screw the part together.
    a. The screws must be turned almost simultaneously. Otherwise, the top part will be off and liquid will spill.
7. Put the N2 hose onto the 6. There is a safe-lock mechanism, it takes a bit of force.
8. Make sure that a thin plastic tube is connected to 2. The thin plastic tube should end up in one of the 15 mL tubes.
9. Take a 5 mL syringe (with orange needle (18G)) and fill the syringe with hydration buffer. Put it in the nozzle on top of 6. Put 7 on 6, this also has a safe-lock.
10. Close the valve on the extruder (8) so pressure can be applied. Put the N2 switch (right) on. Open the black round valve on the left by turning it clockwise ¼. After that, turn the regulator

(middle valve) until fluid comes out of the plastic tube connected to 2. At this point, close the black valve on the left and wait until all the fluid is out.

   a. By doing this, the regulator (middle valve) is set.

11. 9. When all the fluid is out, open the valve on the extruder (8) slightly to let the pressure out. When no more gas comes out (make sure of this!!), close it again and then the cap of the extruder (7) can be removed.
12. Inject the liposome mixture (10 mL max) in the extruder and close it again. Place a new 15 mL tube at the end of the plastic tube at 2. As the regulator settings are already set now, the black valve needs to be opened for only a second for the liposomes to be extruded.
    a. When the system seems clogged, wait patiently. The pressure can be increased by turning the regulator a bit further.
    b. To avoid clogging, a smaller amount of liposomes can be added to the extruder. This is especially important with the later extruder steps.
    c. To avoid clogging, replace the membranes every 4 extrusions.
    d. When no liquid seems to come out after a while, the system is clogged. At this point, release the pressure by opening 8. Open the extruder by removing the cap (7). With a syringe and needle, remove the liposomal mixture from the extruder. After this, carefully remove the 6 from the extruder and remove the excess liposomes seen in the extruder. Replace the membranes and try again.
13. Unscrew the extruder, replace the membranes.
14. Repeat steps 12 for the same membrane combinations
15. Repeat step 12 with different membrane combinations as described in step 4.
16. Unscrew the extruder, throw the membranes away. Clean the parts of the extruder with demi water and with ethanol afterwards.
17. Place the liposomes in a 15 mL tube, label it, and store in the fridge until the next step.

Measuring size of liposomes

Make sure someone has shown how to perform these steps before you do!

1. Go to the **ZetaSizer Nano-S**.
2. Run the test sample. This can be found in 3.30 K1. This contains particles with fixed size and zeta potential to measure.
3. Dilute the liposomes in HBS (pH, 7.4) (Appendix B, DLS & ZP mixtures). Add 30 µL liposomes to 970 µL HBS.
4. Take a cuvette with arrow on the side and fill it with the diluted liposomes in HBS
5. Open the ZetaSizer S with the big button. Place the cuvette with the arrow facing you. Close the lid
6. Open the ZetaSizer software called Malvern
   a. File → new → Measurement file → (your name)
   b. SOP: general purpose
   c. Start (green play button): enter filename here (date/name/liposome etc)
   d. Press the green play button on the new shown page.
7. Now, the measurement has started. For the results:
   a. The derived count rate must be between 20.000 and 140.000. If it is below 20.000, increase the liposome concentration diluted in HBS. If it is too high, dilute the liposomes more.
   b. The attenuator must be between 5 and 11. If it is lower than 4, increase the liposome concentration. If it is too high, dilute the liposomes more.

8. Data is automatically made. Make an excel sheet and place the data in there.
    a. Make sure that the size, PDI, derived count etc are all in the excel sheet
9. Place the liposomes in a 15 mL tube, label it, and store in the fridge until the next step.

Measuring zeta-potential of liposomes

Make sure someone has shown how to perform these steps before you do!

1. Go to the **ZetaSizer Nano-Z**.
2. Run the test sample. This can be found in 3.30 K1. This contains particles with fixed size and zeta potential to measure.
3. Clean the dip cell with a ragger + demi water and ragger + ethanol afterwards.
4. Dilute 30 µL of the test sample with 970 µL10 mM HEPES (pH, 7.5) (see Appendix B, DLS & ZP mixtures).
5. Put the test sample in a cuvette, put the dip cell in the cuvette. Do this by tilting the cuvette while placing the dip cell to remove bubbles.
6. Place the cuvette with dip cell in the ZetaSizer. H2O should be readable.
7. Open the ZetaSizer software called Malvern
    a. File → new → Measurement file → (your name)
    b. SOP: Dip Cell Zeta standard
    c. Start (green play button): enter filename here (date/name/liposome etc)
    d. Press the green play button on the new shown page.
8. Repeat steps 3-7 with the liposomes
9. Data is automatically made. Make an excel sheet and place the data in there.
10. Place the liposomes in a 15 mL tube, label it, and store in the fridge until the next step.

Dialysis 1

Make sure someone has shown how to perform these steps before you do!

1. Make 4 L dialysis 1 buffer (see Appendix B, Dialysis 1). This mixture contains HEPES which is photosensitive. Cover the top of the bucket with tinfoil.
2. Take a Dialysis Cassette 10k MWCO (slide-A-lyzer™, ThermoFisher Scientific) of 3 mL. Place the cassette(s) in the buffer for 2 min. Do this by placing a foam cap on the cassette to make it float.
3. Remove the liposomes from the fridge and place them in the cassette(s). Do this by removing the cap of the cassette (turn the cap so it can be removed), do the liposomes in a syringe and place the content into the cassette.
    a. Remove leftover air in the cassette by softly pushing the membranes. When all the air is out, placed the cap back. By doing this, maximal surface contact is achieved.
4. Take 2 L of dialysis 1 buffer.
5. Put a float cap on top of the cassette(s) and add a magnet into the beaker to stir the solution (convection + diffusion). Take the bucket to the cold room with 4°C (room 3.26), place it on a magnetic board. Stirring speed approximately 1/3 of maximum speed for 2 hours
6. Refresh dialysis with remaining 2 L
7. Dialyse overnight
   - next day -
8. Remove the bucket from the cold room. Remove the cassette(s) from the buffer and with the use of a syringe and needle, remove the content of the cassette from the top of the cassette.
9. Place the liposomes in a 15 mL tube, label it, and store in the fridge until the next step.

Remote loading of L-DOPA

Make sure someone has shown how to perform these steps before you do!

1. Put the water bath of the rotavapor at 60°C.
2. Prepare the levodopa stock solution (0.5 M).
    a. Dilute 1.6 M HCl 7.5 times with demi water to make 1.5 mL (see Appendix B, Levodopa stock solution 0.5 M).
    b. Mix the 1.5 mL HCL with the levodopa and sodium bisulfite (Appendix B, Levodopa stock solution 0.5 M). Sodium bisulfite is added to inhibit early levodopa conversion.
3. Dilute the levodopa stock solution (0.5 M) with dialysis buffer 1 (see Appendix B, Dialysis 1) to produce a 75 mM levodopa stock solution (see Appendix B, Levodopa stock solution 75 mM).
4. Add liposomes to the 75 mM levodopa stock solution (1:2 v/v).
5. Add 10% (v/v) absolute ethanol to the mixture with a positive displacement pipette to enhance liposomal permeability.
6. Take a 15 mL tube. Put the mixture of liposomes, ethanol and levodopa in the tube.
7. Go to the rotavapor, put the tube in one of the connectors. The cap of the 15 mL tube should be stuck in the connector so she tube hangs out.
8. Connect the connector in the rotavapor and make sure that the 15 mL tube is in the water when turning the rotavapor on. Rotate at maximum speed for 2 hours. Turning on the aspirator and increasing pressure is not needed here.
9. When the 2 hours are over, remove the tube from the rotavapor are place is in the cold room (room 3.26) to stop the loading.
10. Start Dialysis 2

Dialysis 2

Make sure someone has shown how to perform these steps before you do!

1. Make 4 L dialysis 2 buffer (see Appendix B, Dialysis 2).
2. Take a Dialysis Cassette 10k MWCO (slide-A-lyzer™, ThermoFisher Scientific) of 3 mL. As 3 mL fits in these cassettes, make sure you take enough. Place the cassette(s) in the buffer for 2 min. Do this by placing a foam cap on the cassette to make it float.
3. Remove the liposomes from the fridge and place them in the cassette(s). Do this by removing the cap of the cassette (turn the cap so it can be removed), do the liposomes in a syringe and place the content into the cassette.
    a. Remove leftover air in the cassette by softly pushing the membranes. When all the air is out, placed the cap back. By doing this, maximal surface contact is achieved.
4. Take 2 L of dialysis 1 buffer.
5. Put a float cap on top of the cassette(s) and add a magnet into the beaker to stir the solution (convection + diffusion). Take the bucket to the cold room with 4°C (room 3.26), place it on a magnetic board. Stirring speed approximately 1/3 of maximum speed for 2 hours.
6. Refresh the dialysis buffer with remaining 2 L.
7. Dialyse overnight
   - next day -
8. Remove the bucket from the cold room. Remove the cassette(s) from the buffer and with the use of a syringe and needle, remove the content of the cassette from the top of the cassette.
9. Place the liposomes in a 15 mL tube, label it, and place in the fridge until the next step.

Liposomal total lipid concentration

Make sure someone has shown how to perform these steps before you do!

1. Make a 0.5mM sodium biphosphate solution. There is a batch in fridge 3.38 Vk2. If it is empty, 50mM sodium biphosphate can be found in fridge 3.38Vk2. Dilute with Milli-Q (see Appendix B, Rouser sodium biphosphate stock solution 0.5 mM).
2. Calculate the amount (mL) of liposomes needed to have a phosphate concentration between 60 and 100 nM (see Appendix B, Rouser lipid calculations).
    a. During the steps preceding Rouser, the lipid concentration changed. After dialysis 2, close to **20%** of the original concentration is left.
3. Switch on both block heaters to 180°C.
4. Grab 10 mL glass tubes. 21 for the standards and 3 for each liposome mixture. Place the glass tubes in a glass tube holder rack.
5. To prepare the standards for the calibration curve, 0, 40, 60, 80, 100, 120 and 140 nM samples are made in triplicates with the use of sodium biphosphate
    a. 0, 80, 120, 160, 200, 240 and 280 µL sodium biphosphate in triplicated
    b. Pipet the amounts directly to the bottom of the glass tubes
    c. Label each glass tube properly
6. To prepare the samples, take the amount of liposomes that should be approximately 60-100 nM, as calculated in step 2.
7. Evaporate the standards and samples in the block heater (180-200C for 30min) until are dry
8. Add 0.3 ml of perchloric acid to each tube with the use of the dispenser in the fume hood.
    a. Put on gloves for this
9. Heat the test tubes to 180C in the block heater for at least 1 hour and max 3 hours. Place marbles on the glass tubes so the content does not evaporate out of the glass tube.
10. During the waiting step, prepare the following solutions:
    a. 1.25% hexa-ammoniummolybdate
        i. Name on shelve is ammoniumheptamolybdate tetrahydrate
        ii. 0.25 gram with 20 mL milli-Q
    b. 5% ascorbic acid
        i. 1.5 gram with 30 mL milli-Q
    c. Fill a beaker with 200 mL Milli-Q
11. Remove the glass tubes from the block heater when the time is over.
12. Add 1 mL Milli-Q to each tube
13. Add 0.5 mL of the hexa-ammoniummolybdate-solution
14. Add 0.5 mL of the ascorbic acid-solution
15. Mix the tubes by vortexing
16. Take a red bin beneath the sink and fill it with hot water. Place the glass tube holder with all the tubes in the hot water for 5 minutes.
17. Remove the hot water from the bin and replace it with cold water. Place the glass tube holder with the tubes in the cold water for 5 minutes.
18. Take a clean F-bottom 96-wells plate. Place 150 µL of each sample in the plate. Make sure to write a lay-out of where each sample it.
    a. Also, fill 3 wells with Milli-Q for the negative control
19. Take the plate to the spectrophotometer (room 3.46). Open the machine, place the plate and close.
20. Open SPECTROstar Nano and choose the phosphate determination paree method to analyse the plate.

21. Open MARS in the SPECTROstar software to export the data to excel.
22. With the use of linear regression, the phospholipid concentration in the liposome batch can be calculated. When the phospholipid concentration is known, the total lipid concentration can be calculated as the ratio of phospholipids and other lipids are known.
23. Place the liposomes in a 15 mL tube, label it, and place in the fridge until the next step.

Liposomal L-DOPA concentration

Make sure someone has shown how to perform these steps before you do!

1. Prepare 500 mL of UPLC Eluent (see Appendix B, UPLC eluent)
2. Check if the 50% MeOh and 50% ACN bottles have at least 300 mL. If not, refill. Both MeOh and ACN can be found in the cabinet in room 3.46.
3. Grab the map next to the UPLC and follow its steps.
4. When the 21 min wait step takes place, prepare the samples and standards
    a. Standards: Levodopa in 0.05% triton-X100 in Milli-Q. the levodopa range ranges for 0 to 3 mM. Prepare the following:

Concentration L-dopa (mM)	mL MQ	mL 3mM stock L-dopa in Milli-Q Triton-X100	Total mL
3	0	1	1
2,5	0.17	0.83	1
2	0.33	0.66	1
1,5	0.5	0.5	1
1	0.66	0.33	1
0,5	0.83	0.17	1
0	1	0	1

    b. Samples: Liposomes in 0.05% triton-X100 in Milli-Q. Prepare the following:

Dillution factor	mL liposomes	mL Milli-Q Triton-X100	Total mL
2x	0.3	0.3	0.6
5x	0.2	0.8	1
10x	0.1	0.9	1
20x	0.05	0.95	1
50x	0.02	0.98	1
100x	0.01	0.99	1

5. Place 200 µL of the standards in UPLC vials with blue caps, do this is triplicate. Place the vials in a UPLC vials holder, lie next to the UPLC machine. Make a lay-out of the vials.
6. Place 200 µL of the samples in UPLC vials with blue caps, do this in triplicate. Place the vials in a UPLC vials holder, lie next to the UPLC machine. Make a lay-out of the vials.
7. When the time is over. Check the map and install the column (HSS T3 1,8um, 2.1mm x 50mm, Waters).
    a. Open the little door where the column must be placed. Remove the metal part by twisting the rubber part loose, after that the metal one. Replace the metal part with the UPLC column. The flow goes to the left.
8. Open the UPLC machines door and place the UPLC vial holder in the machine
9. Follow the map again.
    a. Methods, 100A1_5min@OP3_UV (folder, Marcel Fens)
    b. Injection volume: 5 µL

    c. Flow rate eluent, 0,3 mL/min
    d. Wavelengths for detection: 280 nm
    e. Sample rate: 40 points/sec
    f. Runtime (initial): 2 min
10. Before starting the measurement, equilibrate for 10 minutes. The map tells how to do this.
11. Start the run. The map tells how to do this.
    a. Make sure to fill in the shutting down method.
12. Analyse the data at the workstation computers next to the technicians. The data is stored there.
13. Use Empower chromatography data system to analyse the Area Under the Curve of the UPLC peaks. With the use of linear regression, the levodopa concentration in the liposomes can be calculated. The map at the computers explains how to do this. If not clear, ask technicians.
14. Place the liposomes in a 15 mL tube, label it, and place in the fridge until the next step.

Ultracentrifuge (optional)

Make sure someone has shown how to perform these steps before you do!

1. Grab polycarbonated tubes. These are 10mL. Because equal weight is crucial with UC, make sure you have an even number of tubes.
2. Fill the tubes. If you have a sample of 3 mL, fill it up with dH2O until 10mL. This fills the tube almost fully. Weight this tube. Now fill the second tube also almost to the top with either sample with dH2O or only dH2O. Weight this tube.
    a. The weight difference between the tubes must **NEVER** be more than 0.1 gram. Try to get it below 0.05 just to be sure.
3. Start the UC with the power switch that is located on the right side of the machine.
4. Open the rotor compartment. Don't pull hard or anything, it goes smoothly.
5. Grab the rotor and place your tubes in on opposite sides.
6. Close the rotor with the red cap. Close it with the screw with your hand. When you cannot turn it further, grab the "tube removal tool" to turn it a bit tighter than hand tight
7. Lift up the rotor a place it in the machine. Make sure that while you lift, you keep the rotor above the machine and not above the floor. This way, if you let it slip, damage will be less costly.
    a. The rotor will not be locked yet. Gif it a swirl and it must turn
8. Close the rotor compartment
9. Choose the right program
    a. Acceleration and deceleration must be slow.
    b. 4°C, 55000g for 1 hour
10. Start the program by pressing enter and then quickly press start
11. Fill in the logbook
12. When the run is done, the vacuum needs to be released manually. Do this by pressing the vacuum button. This only works when the R.P.M. is below 3000. This takes roughly one minute. When the sound that comes free with releasing the vacuum ends, wait another 10 seconds. When R.P.M reaches 0, the rotor compartment can be opened.
    a. **NEVER** try to open compartment when the UC is still going or is under pressure. It is not possible and the handle will be damaged if you try it.
13. Remove the rotor from the compartment. unscrew the screw with the tube removal tool and remove the cap. Remove the tubes. Place the rotor upside down, with the cap etc. off, so condense fluids can leak out.
    a. If the rotor is dirty, clean it with water and soap (with tissues). Afterwards, do the same with ethanol. The rotor must be placed upside down again afterwards so that the fluids

can get out of it. Dry the rotor as much as you can at titanium and fluids do not go well together.

14. Fill in the "revolution counter" in the logbook when you are done. This can be found on the left side of the operating panel.
15. Turn of the UC by pressing the red emergency button located right below the operating panel. Leave the rotor compartment open so condense can leave.
16. Remove the supernatant and keep that in a tube as well. Label both the supernatant as the pellet as it is interesting to look at the L-dopa level in both.
17. Resuspend the pellet with HBS and place both the supernatant as the pellet in the fridge.
18. Place the liposomes in a 15 mL tube, label it, and place in the fridge until the next step.

Confirming the bond between biotinylated liposomes and magnetic streptavidin beads.

1. Make the (non-)biotinylated liposomes with protocols "Rotavapor" and "Extrusion" and Appendix B, Lipid mixture for liposome preparation for the lipid mixtures. Extrude the liposomes until 80 nm liposomes are made for:
    - **a.** Batch A: liposomes with biotin (1% of PEG)
    - **b.** Batch B: liposomes without biotin (negative control)
2. With the ZetaSizer Nano-S/Z, measure the size and the zeta potential of the produced liposomes. Use the protocols "Measuring size of liposomes" and "Measuring zeta-potential of liposomes" to do this. Make sure that the size is close to the aimed 80 or 120nm.
3. To remove the excess ammonium sulfate from the hydration buffer used while making the liposomes, perform dialysis as described in preparation protocol "Dialysis 1".
4. Load the liposomes with L-DOPA with protocol "Remote loading of the liposomes and Dialysis 2". With this protocol, excess L-DOPA is also removed.
5. To determine the total lipid and levodopa concentration in the batch, use the characterisation protocols "Liposomal total lipid concentration" and "Liposomal L-DOPA concentration" for this.
6. Grab 4 Eppendorf tubes with the round bottom (2mL) for in the magnetic rack and label them appropriately. Example:
    - **a.** A1, 80 nm DiD-labelled biotinylated liposomes
    - **b.** B1, 80 nm DiD-labelled biotinylated liposomes
    - **c.** A2, 80 nm DiD-labelled non-biotinylated liposomes
    - **d.** B2, 80 nm DiD-labelled non-biotinylated liposomes
7. Grab 24 round bottom Eppendorf tubes and name them. Example:
    - **a.** A1S, A1S2, A1S3, S stands for supernatant
    - **b.** A1P, A1P2 A1P3, P stands for pellet
        i. This is repeated for B1, A2 and B2
8. For tubes A1, B1, A2 and B2: add 500 µL of the magnetic streptavidin beads.
9. Wash the beads in each tube by adding 1 mL PBS, vortex the tube and placing the tube in the magnetic rack. After a minute, a pellet of beads formed. The supernatant can be removed. Repeat this twice more.
10. Add 100 µL PBS to the magnetic streptavidin beads.
11. Add 450 µM 80 nm liposomes, as mentioned in step 5.
12. Place the Eppendorf tubes in a 50 mL tube. Place the 50 mL tubes in the cold room on the rocker for 10 minutes.

13. After the incubation, remove the tubes from the cold room and place the Eppendorf tubes in the magnetic rack. After a minute, a pellet formed. Remove the supernatant and place in the corresponding Eppendorf tubes: A1S, B1S, A2S and B2S
14. Wash Eppendorf tubes A1, B1, A2 and B2 twice more with 100 µL and collect the supernatant in the corresponding Eppendorf tubes: 6a, A1S2&3, B1S2&3, A2S2&3 B2S2&3.
15. Add 100 µL DMF in the tubes of step 12 and 13.
16. Add 100 µL DMF in tubes A1, B1, A2 and B2. Mix the content with vortex and place the tubes in the magnetic rack.
17. When the pellets occurred, relocate the supernatant in the corresponding tubes: A1P, B1P, A2P and B2P.
18. Wash Eppendorf tubes A1, B1, A2 and B2 twice more with 100 µL DMF and collect the supernatant in the corresponding Eppendorf tubes: 6b, A1P2&3, B1P2&3, A2P2&3 and B2P2&3.
19. Add 100 µL PBS in the Eppendorf tubes of step 15 and 16.
20. Place 200 µL of each tube of step 6 in a black F-bottom 96-Well plate.
21. Take the 96-Well plate to the JASCO FP-8300 Fluorescence Spectrometer to measure the DiD signal in the samples.

JASCO FP-8300 Fluorescence Spectrometer

Make sure someone has shown how to perform these steps before you do!

1. Install the needed accessory to the JASCO. There is one for 1-4 cuvettes, and one for a 96-Well plate. Take the 96-Well one. A map is present at the JASCO that gives instructions on how to do this.
2. Switch one the computer and JASCO (switch on the left side of the machine)
3. Open SpectraManager and wait until the initialisation is finished and is changed to IDLE.
4. As the DiD-label is measured at ex: 648 nm and em: 670 nm, choose fixed wavelengths measurements
5. Click settings at the left bottom of the screen and click initialising afterwards. The machine will now place the plate holder to the correct location in the machine for you to place the Well-plate in. If not, turn of the software and machine and turn it on again.
6. Press the button below. Calculate how many samples are measured.

7. Press the button showed below.
    a. 
    b. The JASCO is not able to skip Wells, so place the samples accordingly.
8. Press the button showed below.
    a. 
    b. General: fill in the excitation (648 nm) and emission (670 nm) wavelengths.
    c. General: sensitivity: medium
    d. General: bandwidth: 5 nm
        i. Both sensitivity and bandwidth can be adjusted to increase quality of results if needed. By increasing sensitivity, more signal will be measured (also background!). By decreasing bandwidth, more precise measurements will be performed
9. Open de ex-shutter for measurements: no
10. Make sure that both the excitation and emission shutters are open as shown in the Figure below:

11. 
12. Open the lid of the JASCO and place the 96-Well plate, A1 in top left corner
13. Start the measurement by clicking:
    a. 
14. Edit, copy all, open excel sheet, copy data.

Time determination for biotin-streptavidin interaction

1. Make the biotinylated liposomes with protocols “Rotavapor” and “Extrusion” and Appendix B, lipid mixture for liposome preparation for the lipid mixture. Extrude the liposomes until 80 nm liposomes are made for:
    a. Batch A: liposomes with biotin (1% of PEG)
2. With the ZetaSizer Nano-S/Z, measure the size and the zeta potential of the produced liposomes. Use the protocols “Measuring size of liposomes” and “Measuring zeta-potential of liposomes” to do this. Make sure that the size is close to the aimed 80 nm.
3. To remove the excess ammonium sulfate from the hydration buffer used while making the liposomes, perform dialysis as described in preparation protocol “Dialysis 1”.
4. Load the liposomes with L-DOPA with protocol “Remote loading of the liposomes and Dialysis 2”. With this protocol, excess L-DOPA is also removed.
5. To determine the total lipid and levodopa concentration in the batch, use the characterisation protocols “Liposomal total lipid concentration” and "Liposomal L-DOPA concentration" for this.
6. Grab 6 Eppendorf tubes with the round bottom (2mL) for in the magnetic rack and label them appropriately. Example:
    a. A1, 80 nm DiD-labelled biotinylated liposomes, incubated for 2.5 min
    b. B1, 80 nm DiD-labelled biotinylated liposomes, incubated for 2.5 min
    c. A2, 80 nm DiD-labelled biotinylated liposomes, incubated for 5 min
    d. B2, 80 nm DiD-labelled biotinylated liposomes, incubated for 5 min
    e. A3, 80 nm DiD-labelled biotinylated liposomes, incubated for 10 min
    f. B3, 80 nm DiD-labelled biotinylated liposomes, incubated for 10 min
7. Grab 18 round bottom Eppendorf tubes and name them. Example:
    a. A1P, A1P2, A1SP, P stands for Pellet
        i. This is repeated for B1, A2, B2, A3 and B3.
8. For tubes A1, B1, A2, B2, A3 and B3: add 275 µL of the magnetic streptavidin beads.
9. Wash the beads in each tube by adding 1 mL PBS, vortex the tube and placing the tube in the magnetic rack. After a minute, a pellet of beads formed. The supernatant can be removed. Repeat this twice more.
10. Add 100 µL PBS to the magnetic streptavidin beads.
11. Add 500 µM 80 nm liposomes, as mentioned in step 5.
12. Place the Eppendorf tubes in a 50 mL tube. Place the 50 mL tubes in the cold room on the rocker for 10 minutes.
13. After the incubation, remove the tubes from the cold room and place the Eppendorf tubes in the magnetic rack. After a minute, a pellet formed. Remove the supernatant.
14. Wash Eppendorf tubes twice more with 100 µL.
15. Add 100 µL DMF in the Eppendorf tubes.
16. Add 100 µL DMF in tubes A1, B1, A2, B2, A3 and B3. Mix the content with vortex and place the tubes in the magnetic rack.
17. When the pellets occurred, relocate the supernatant in the corresponding tubes: A1P, B1P, A2P , B2P, A3P and B3P.

18. Wash Eppendorf tubes A1, B1, A2, B2, A3 and B3 twice more with 100 µL DMF and collect the supernatant in the corresponding Eppendorf tubes: 6b, A1P2&3, B1P2&3, A2P2&3, B2P2&3, A3P2&3 and B3P2&3.
19. Add 100 µL PBS in the Eppendorf tubes of step 17 and 18.
20. Place 200 µL of the tubes of step 17 and 18 in a black F-bottom 96-Well plate.
21. Take the 96-Well plate to the JASCO FP-8300 Fluorescence Spectrometer to measure the DiD signal in the samples.

Determination of the point of saturation for the magnetic streptavidin beads

1. Make the biotinylated liposomes with protocols “Rotavapor” and “Extrusion” and Appendix B, Lipid mixture for liposome preparation for the lipid mixture. Extrude the liposomes until 80 and 120 nm liposomes are made for:
    a. Batch A: liposomes with biotin (1% of PEG)
2. With the ZetaSizer Nano-S/Z, measure the size and the zeta potential of the produced liposomes. Use the protocols “Measuring size of liposomes” and “Measuring zeta-potential of liposomes” to do this. Make sure that the size is close to the aimed 80 and 120 nm.
3. To remove the excess ammonium sulfate from the hydration buffer used while making the liposomes, perform dialysis as described in preparation protocol “Dialysis 1”.
4. Load the liposomes with L-DOPA with protocol “Remote loading of the liposomes and Dialysis 2”. With this protocol, excess L-DOPA is also removed.
5. To determine the total lipid and levodopa concentration in the batch, use the characterisation protocols “Liposomal total lipid concentration” and "Liposomal L-DOPA concentration" for this.
6. Grab 20 Eppendorf tubes with the round bottom (2mL) for in the magnetic rack and label them appropriately. Example:
    a. A1, 100 µM 80 nm DiD-labelled biotinylated liposomes
    b. B1, 100 µM 80 nm DiD-labelled biotinylated liposomes
    c. A2, 150 µM 80 nm DiD-labelled biotinylated liposomes
    d. B2, 150 µM 80 nm DiD-labelled biotinylated liposomes
    e. A3, 200 µM 80 nm DiD-labelled biotinylated liposomes
    f. B3, 200 µM 80 nm DiD-labelled biotinylated liposomes
    g. A4, 250 µM 80 nm DiD-labelled biotinylated liposomes
    h. B4, 250 µM 80 nm DiD-labelled biotinylated liposomes
    i. A5, 300 µM 80 nm DiD-labelled biotinylated liposomes
    j. B5, 300 µM 80 nm DiD-labelled biotinylated liposomes
        i. This is repeated for 120 nm liposomes
7. Grab 60 round bottom Eppendorf tubes and name them. Example:
    a. A1S, A1S2, A1S3, S stand for supernatant
    b. A1P, A1P2, A1SP, P stands for Pellet
        i. This is repeated for B1, A2, B2, A3, B3, A4, B4, A5 and B5.
        ii. This is repeated for the 120 nm liposomes samples
8. For tubes A1, B1, A2, B2, A3, B3, A4, B4, A5 and B5: add 275 µL of the magnetic streptavidin beads.
9. Wash the beads in each tube by adding 1 mL PBS, vortex the tube and placing the tube in the magnetic rack. After a minute, a pellet of beads formed. The supernatant can be removed. Repeat this twice more.
10. Add 500 µL PBS to the magnetic streptavidin beads.
11. Add µM 80 nm liposomes, as mentioned in step 5, in the corresponding Eppendorf tubes.

12. Place the Eppendorf tubes in a 50 mL tube. Place the 50 mL tubes in the cold room on the rocker for 10 minutes.
13. After the incubation, remove the tubes from the cold room and place the Eppendorf tubes in the magnetic rack. After a minute, a pellet formed. Remove the supernatant and place in the corresponding Eppendorf tubes: A1S, B1S, A2S, B2S, A3S, B3S, A4S, B4S, A5S and B5S.
14. Wash Eppendorf tubes A1, B1, A2, B2, A3, B3, A4, B4, A5 and B5 twice more with 500 µL PBS and collect the supernatant in the corresponding Eppendorf tubes: A1S2&3, B1S2&3, A2S2&3 B2S2&3, A3S2&3, B3S2&3, A4S2&3, B4S2&3, A5S2&3 and B5S2&3
15. Add 500 µL DMF in the tubes of step 12 and 13.
16. Add 500 µL DMF in tubes A1, B1, A2, B2, A3, B3, A4, B4, A5 and B5. Mix the content with vortex and place the tubes in the magnetic rack.
17. When the pellets occurred, relocate the supernatant in the corresponding tubes: A1P, B1P, A2P, B2P, A3P, B3P, A4P, B4P, A5P and B5P.
18. Wash Eppendorf tubes A1, B1, A2, B2, A3, B3, A4, B4, A5 and B5 twice more with 500 µL DMF and collect the supernatant in the corresponding Eppendorf tubes: A1P2&3, B1P2&3, A2P2&3 B2SP&3, A3P2&3, B3P2&3, A4P2&3, B4P2&3, A5P2&3 and B5P2&3
19. Add 500 µL PBS in the Eppendorf tubes of step 16 and 17.
20. Place 200 µL of each tube of step 6 in a black F-bottom 96-Well plate.
21. Take the 96-Well plate to the JASCO FP-8300 Fluorescence Spectrometer to measure the DiD signal in the samples.

Streptavidin recycling

1. Make the biotinylated liposomes with protocols “Rotavapor” and “Extrusion” and Appendix B, lipid mixture for liposome preparation for the lipid mixture. Extrude the liposomes until 80 nm liposomes are made for:
    a. Batch A: liposomes with biotin (1% of PEG)
2. With the ZetaSizer Nano-S/Z, measure the size and the zeta potential of the produced liposomes. Use the protocols “Measuring size of liposomes” and “Measuring zeta-potential of liposomes” to do this. Make sure that the size is close to the aimed 80 nm.
3. To remove the excess ammonium sulfate from the hydration buffer used while making the liposomes, perform dialysis as described in preparation protocol “Dialysis 1”.
4. Load the liposomes with L-DOPA with protocol “Remote loading of the liposomes and Dialysis 2”. With this protocol, excess L-DOPA is also removed.
5. To determine the total lipid and levodopa concentration in the batch, use the characterisation protocols “Liposomal total lipid concentration” and "Liposomal L-DOPA concentration" for this.
6. Grab 12 Eppendorf tubes with the round bottom (2mL) for in the magnetic rack and label them appropriately. Example:
    a. A1, 80 nm DiD-labelled biotinylated liposomes, not heated
    b. B1, 80 nm DiD-labelled biotinylated liposomes, not heated
    c. A2, 80 nm DiD-labelled biotinylated liposomes, heated to 70°C (1 sec)
    d. B2, 80 nm DiD-labelled biotinylated liposomes, heated to 70°C (1 sec)
    e. A3, 80 nm DiD-labelled biotinylated liposomes, heated to 70°C (30 sec)
    f. B3, 80 nm DiD-labelled biotinylated liposomes, heated to 70°C (30 sec)
    g. A4, 80 nm DiD-labelled biotinylated liposomes, heated to 70°C (60 sec)
    h. B4, 80 nm DiD-labelled biotinylated liposomes, heated to 70°C (60 sec)
    i. A5, 80 nm DiD-labelled biotinylated liposomes, heated to 70°C (600 sec)
    j. B5, 80 nm DiD-labelled biotinylated liposomes, heated to 70°C (600 sec)
    k. A6, 80 nm DiD-labelled biotinylated liposomes, heated before incubation
    l. B6, 80 nm DiD-labelled biotinylated liposomes, heated before incubation

7. Grab 36 round bottom Eppendorf tubes and name them. Example:
    a. A1P, A1P2, A1SP, P stands for Pellet
    b. This is repeated for B1, A2, B2, A3, B3, A4, B4, A5, B5, A6 and B6.
8. For tubes A1, B1, A2, B2, A3, B3, A4, B4, A5, B6, A6 and B6: add 275 µL of the magnetic streptavidin beads.
9. Wash the beads in each tube by adding 1 mL PBS, vortex the tube and placing the tube in the magnetic rack. After a minute, a pellet of beads formed. The supernatant can be removed. Repeat this twice more.
10. Add 500 µL PBS to the magnetic streptavidin beads.
11. Place a normal rack and the magnetic rack in the warmth bath and place A6 and B6 in the rack. Make sure that, even though the rack must be in water, the top of the Eppendorf tubes cannot be under water.
12. Heat up the warmth batch to 70°C for 600 sec.
13. Remove A6 and B6 from the normal rack and place in the magnetic rack. Use a tong as the water is hot.
14. The pellet will form in a minute. Remove the supernatant. Remove the Eppendorf tubes from the water bath and place them in a rack. Remove the magnetic rack from the water bath as well. Again, use tong as the water is hot. Let the water bath and magnetic rack cool down.
15. Add 500 µL PBS to the magnetic streptavidin beads of A6 and B6.
16. Add 120 µM 80 nm liposomes to A1, B1, A2, B2, A3, B3, A4, B4, A5, B6, A6 and B6.
17. Place the Eppendorf tubes in a 50 mL tube. Place the 50 mL tubes in the cold room on the rocker for 10 minutes.
18. After the incubation, remove the tubes from the cold room and place the Eppendorf tubes in the magnetic rack. After a minute, a pellet formed. Remove the supernatant.
19. Wash Eppendorf tubes A1, B1, A2, B2, A3, B3, A4, B4, A5 and B5 twice more with 500 µL PBS.
20. Add 500 µL DMF in tubes A1, B1, A2, B2, A3, B3, A4, B4, A5, B5, A6 and B6. Mix the content with vortex and place the tubes in the magnetic rack.
21. When the pellets occurred, relocate the supernatant in the corresponding tubes: A1P, B1P, A2P, B2P, A3P, B3P, A4P, B4P, A5P, B5P, A6P and B6P.
22. Wash Eppendorf tubes A1, B1, A2, B2, A3, B3, A4, B4, A5, B5, A6 and B6 twice more with 500 µL DMF and collect the supernatant in the corresponding Eppendorf tubes: A1P2&3, B1P2&3, A2P2&3 B2SP&3, A3P2&3, B3P2&3, A4P2&3, B4P2&3, A5P2&3, B5P2&3, A6P2&3 and B6P2&3.
23. Add 500 µL PBS in the Eppendorf tubes of step 20 and 21.
24. Place 200 µL of each tube of step 23 in a black F-bottom 96-Well plate.
25. Take the 96-Well plate to the JASCO FP-8300 Fluorescence Spectrometer to measure the DiD signal in the samples.
26. Place A1, B1, A2, B2, A3, B3, A4, B4, A5, B6, A6 and B6 in the magnetic rack and after a minute, remove the supernatant.
27. Add 500 µL Milli-Q to each sample, except for A1 and B1. Place A2-B6 in the normal rack the warm bath and heat up as specified in step 6.
28. When the time is over, place the Eppendorf tubes from the normal rack in the water bath to the magnetic rack, wait a minute and remove the supernatant.
29. Repeat step 10, 16-25.

Retention studies

Biological media refers to HBS (pH, 6.5), blood plasma and whole blood.

1. Make the biotinylated liposomes with protocols “Rotavapor” and “Extrusion” and Appendix B, lipid mixture for liposome preparation for the lipid mixture. Extrude the liposomes until 120 nm liposomes are made for:

   a. Batch A: liposomes with biotin (1% of PEG)
2. With the ZetaSizer Nano-S/Z, measure the size and the zeta potential of the produced liposomes. Use the protocols “Measuring size of liposomes” and “Measuring zeta-potential of liposomes” to do this. Make sure that the size is close to the aimed 120 nm.
3. To remove the excess ammonium sulfate from the hydration buffer used while making the liposomes, perform dialysis as described in preparation protocol “Dialysis 1”.
4. Load the liposomes with L-DOPA with protocol “Remote loading of the liposomes and Dialysis 2”. With this protocol, excess L-DOPA is also removed.
5. To determine the total lipid and levodopa concentration in the batch, use the characterisation protocols “Liposomal total lipid concentration” and "Liposomal L-DOPA concentration" for this.
6. Grab 14 Eppendorf tubes with the round bottom (2mL) for in the magnetic rack and label them appropriately. Example:
   a. A1, 120 nm DiD-labelled biotinylated liposomes, T=0h
   b. B1, 120 nm DiD-labelled biotinylated liposomes, T=0h
   c. A2, 120 nm DiD-labelled biotinylated liposomes, T=1h
   d. B2, 120 nm DiD-labelled biotinylated liposomes, T=1h
   e. A3, 120 nm DiD-labelled biotinylated liposomes, T=2h
   f. B3, 120 nm DiD-labelled biotinylated liposomes, T=2h
   g. A4, 120 nm DiD-labelled biotinylated liposomes, T=4h
   h. B4, 120 nm DiD-labelled biotinylated liposomes, T=4h
   i. A5, 120 nm DiD-labelled biotinylated liposomes, T=6h
   j. B5, 120 nm DiD-labelled biotinylated liposomes, T=6h
   k. A6, 120 nm DiD-labelled biotinylated liposomes, T=24h
   l. B6, 120 nm DiD-labelled biotinylated liposomes, T=24h
   m. A7, 120 nm DiD-labelled biotinylated liposomes, T=48h
   n. A7, 120 nm DiD-labelled biotinylated liposomes, T=48h
7. For tubes A1, B1, A2, B2, A3, B3, A4, B4, A5, B6, A6, B6, A7 and B7: add 275 µL of the magnetic streptavidin beads.
8. Wash the beads in each tube by adding 1 mL PBS, vortex the tube and placing the tube in the magnetic rack. After a minute, a pellet of beads formed. The supernatant can be removed. Repeat this twice more.
9. Add 250 µM 120 nm liposomes in the Eppendorf tubes.
10. Place the Eppendorf tubes in a 50 mL tube. Place the 50 mL tubes in the cold room on the rocker for 10 minutes.
11. After the incubation, remove the tubes from the cold room and place the Eppendorf tubes in the magnetic rack. After a minute, a pellet formed. Remove the supernatant.
12. Wash the Eppendorf tubes with 1 mL PBS.
13. Add 500 µL biological media
   a. For whole blood, add CPDA-1 (1:9, v/v)
   b. For T=0, adding biological media is not needed. Go to step 17.
14. Place the Eppendorf tubes in a 50 mL tube and place that tube in a 1 L glass bottle. The 50 mL tube is stuck in the bottle neck. When placing the bottle on its side and rolling it, the liquid inside the Eppendorf tubes can be seen. Check if the liquid moves. If not, remove the Eppendorf tubes from the 50 mL tube and place them in a bigger angle. Repeat the step.
15. Place the glass 1 L bottle with Eppendorf tubes in a 50 mL tube in the 37°C incubator. Time as stated in step 6.
16. Remove the bottle with Eppendorf tubes from the incubator when the time is over. Remove the Eppendorf tubes and place them in the magnetic rack. After a minute, remove the supernatant.
17. Wash the tubes twice with 1 mL of PBS

18. Add 200 µL Milli-Q 0.05% Triton-X100
19. Place the Eppendorf in the magnetic rack. Place supernatant in UPLC vial with blue cap
20. Measure AUC of L-DOPA concentration with UPLC.