Module 3. Preparation of liposomes and their use in flow cytometry

Objective: Make Siglec-F targeted and Naked liposomes and test their binding to individual Siglec-expressing CHO cells using flow cytometry.

References:

• Chen WC et al, Blood, Vol. 115, pp4778-, 2010

Reagents and equipments:

• Lipid components
  • 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC, Avanti #850365)
  • Cholesterol (Sigma-Aldrich #C8667)
  • N-(Carbonyl-methoxypolyethyleneglycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine (PEG-DSPE, NOF # SUNBRIGHT DSPE-020CN)
  •CN146-PEG-DSPE
• Liposome extruder set (Avanti, #610000)
• Filter support (Avanti # 610014)
• Polycarbonate membranes (Avanti #610005 for 100 nm, 610007 for 400 nm)
• Siglec-F, Siglec-8, and Siglec-15 and parental CHO cells
• HBSS + 0.01%BSA + 2mM EDTA (FACS buffer)
• PBS without Ca and Mg
• Glass tube (Fisher #14-958D)
• FACS tube (BD Biosciences #352052)

Instruments:

• Sonication bath (BRANSON ultrasonic bath 1510-MTH)
• Flow cytometer (FACS calber, BD Biosciences)
• (Optional) Particle size analyzer (Zeta sizer, Malvern)

Method:

1. Liposome preparation:

• Preparation of lipid components (1μmol total lipids)

DSPC (MW=790)	Chol (MW=387)	PEG-DSPE (MW=2900)	CN146-PEG-DSPE (MW=3980)	AF647-DSPE (MW=1763)
57mol%	38mol%	5-Xmol%	Xmol%	1mol%
0.57 μmol	0.38 μmol	0.05-0.0X μmol	0.0X μmol	0.01 μmol

  • Dissolve the DSPC, Chol, and PEG-DSPE in Chloroform (CN146-PEG-DSPE is in DMSO).
  • Add lipid components (except CN146-PEG-DSPE and AF647-DSPE) in glass tube.
  • Vortex the tube.
  • Dry the chloroform with N_2 gas flow
  • Add 200 μL of DMSO. Add CN146-PEG-DSPE and AF647-DSPE
  • Lyophilize the DMSO for 18 h.

Shiteng will prepare the dried lipid components beforehand.

- Hydration of lipid components
  - Add 1 mL of PBS into glass tubes (liposome concentration is 1 mM).
  - Sonicate the solution for 5*30sec with holding the glass tube.

- Extrusion of liposomes
  - Set up the extruder according the following link.
    http://avantilipids.com/index.php?option=com_content&view=article&id=531&Itemid=295
  - Pass the membrane filter gently over 20 times using 400 nm and 100 nm pore size filters.
  - (Optional) Measure the size of liposomes with the light scattering analyzer zetasizer.

2. Analysis of liposome binding to Siglec expressing CHO cells:
- Staining of the cells with liposomes

  Cells (1-3 x 10^5 cells in 100 μL) in 96 Well U bottom plates (Shiteng will prepare in advance)
  - Add 100 μL of liposome solution (Dilution of liposome will be determined by Shiteng in advance).
  - Incubate the cells for 30 min at 37˚C.
  - Wash the cells by adding 100 μL of FACS buffer
  - Spin down the tubes at 300 x g for 5 min.
  - Discard the supernatants.
  - Vortex the tube to loosen the cell pellet.
  - Suspend the cells in 200 μL of FACS buffer containing 1 μg/mL propidium iodide and transfer the cells into the FACS tubes.
  - Vortex the tube well.

- Analyze the liposome binding by FACS Caliber.
  - Turn on the FACS caliber.
  - Turn on the computer.
  - Open the Cell quest software.
  - Make a dot plot of FSC vs SSC (This is for cell detection).
  - Make a dot plot of FSC vs PerCP (This is for dead cell exclusion).
  - Make a histogram plot of AF-647 (This is for liposome binding detection).
  - Make a folder to save the acquired files.
  - Acquire each sample.

  For further information of FACS Caliber, please download the tutorials from the link below.
  http://www.bdbiosciences.com/support/resources/facscalibur/index.jsp

- Analyze the data with Flowjo software.
  - Open the Flowjo software.
  - Drag and drop the folder containing the acquired samples on the flowjo.

• Open the file of “unstained cells” and make a gate to identify the cells on the FSC vs SSC plot.
• Open the gated population and make a dot plot of FSC vs PerCP
• Make a new gate for the living cells (PerCP low).
• Open the gated population and make the histogram plot of APC
• Drag the newly generated files on the “all samples”. Flowjo will automatically make the same gate and histogram plot for all the samples.
• Open the work space in flowjo to draw the histogram data.
• Drag and drop the APC histogram of “unstained cells”.
• To make a overlay histogram, drag and drop the “naked” and “Siglec-F targeted” files on the histogram of “unstained cells”.
For further information of Flowjo, please download the tutorials from the link below.
http://www.flowjo.com/home/tutorials/