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

Objective:

Make human CD22 targeted and Naked liposomes and to test their binding to human CD22 expressing CHO cells using flow cytometry.

References:

Chen WC et al, In vivo targeting of B-cell lymphoma with glycan ligands of CD22. *Blood*. 2010, 115(23): 4778–4786.

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)
    - 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (ammonium salt) (NBD-PE, Avanti #810145C)
    - Human CD22 glycan ligand-PEG-DSPE
- Liposome extruder set (Avanti, #610000)
- Filter support (Avanti # 610014)
- Polycarbonate membranes (Avanti #610005 for 100 nm, 610007 for 400 nm)
- Human CD22-CHO and parental CHO cells
- HBSS + 0.01%BSA (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)

Methods:

1. Liposome preparation:

• Preparation of lipid components (1mmol total lipids)

DSPC (MW=790)	Chol (MW=387)	PEG-DSPE (MW=2900)	CD22 ligand-PEG-DSPE (MW=3880)	NBD-PC (MW=856)
56mol%	38mol%	5-Xmol%	Xmol%	1mol%
0.56 mmol	0.38 mmol	0.05-0.0X mmol	0.0X mmol	0.01 mmol

• Dissolve the DSPC, Chol, PEG-DSPE, and NBD-PC in Chloroform (CD22 ligand-PEG-DSPE is in DMSO).

• Add lipid components (except CD22 ligand) in glass tube.

• Vortex the tube.

• Dry the chloroform with N_2 gas flow.

• Add CD22 ligand-PEG-DSPE in DMSO.

• Lyophilize the DMSO for 18 h.

Norihito will prepare the dried lipid components before the day.

• Hydration of lipid components

• Add 1 mL of PBS into glass tubes (liposome concentration is 1 mM).

• Sonicate the solution for 3 min with holding the glass tube.

• Vortex the tube occasionally.

• 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 human CD22-CHO cells:

• Staining of the cells with liposomes

Cells (1-3 x 10^5 cells in 100 mL) in FACS tube **(Norihito will prepare in advance)**

- Add 100 mL of liposome solution (**Dilution of liposome will be determined by Norihito in advance).**
- Vortex the FACS tube gently.
- Incubate the cells for 30 min at 37˚C.
- Wash the cells by adding 2 mL of FACS buffer (No need for pipetting).
- Spin down the tubes at 510 x g for 3 min.
- Discard the supernatants by pouring the supernatants into waste.
- Vortex the tube to loosen the cell pellet.
- Suspend the cells in 300 mL of FACS buffer containing 1 mg/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 FL3 (This is for dead cell exclusion).
- Make a histogram plot of FL1 (This is for NBD 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 FL3.

- Make a new gate for the living cells (FL3 low).
- Open the gated population and make the histogram plot of FL-1.
- 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 FL-1 histogram of “unstained cells”.
- To make a overlay histogram, drag and drop the “naked” and “CD22-tageted” files on the histogram of “unstained cells”.

For further information of Flowjo, please download the tutorials from the linke below.
http://www.flowjo.com/home/tutorials/

- Make slides in PowerPoint.
  - Copy and paste the overlay histogram on powerpoint file.
  - Make a figure legend.