Liposome synthesis

Compiled by K.V.

AIM:

This protocol aims to describe the steps needed for a successful synthesis of cell-sized monodisperse liposomes. While any custom material might be chosen for interior and exterior solutions, this particular protocol is adjusted for encapsulation of transcriptional translational systems for a successful synthesis of proteins inside the liposomes.

REAGENTS USED:

Name of reagent	**Concentration**	
Ethanol	96%	OA phase
Pluronic P188	10%	
Glucose	2M	
ATP, GTP, CTP, UTP	4 mM, 2 mM	
Spermidine	4 mM	
Creatine phosphate	40 mM	
DTT	2 mM	
10-formyl-5,6,7,8-tetrahydrofolic acid	20 mg/mL	
Potassium glutamate	200 mM	
$Mg(OAc)_2$	26 mM	
HEPES-KOH (pH 7.6)	100 mM	
Fluorescein		
Sucrose	2M	IA phase
PURExpress 2.1	2 x	
Template DNA	0.5 – 3.0 ng/µL per 1 kbp	
RNAse inhibitor	40 U	
Lipids	100 mg/mL	LO phase
2-octanol		
Rh PE	1:1000 [Rh PE:DOPC]	
ddH_2O	100%	OA/IA phases
Glycerol	100%	

Tab 1. List of reagents used in the experiment

Name of a tool	Quantity
Plastic 1 mL syringe	1
Hamilton glass 50 µL syringe	1
Hamilton glass 100 µL syringe	1
Syringe needle	3
Tubing	
Tweezers	
Razor blade	

Tab 2. List of tools used in the experiment

EXPERIMENT DESCRIPTION:

An experiment is conducted using an octanol-assisted liposome assembly (OLA) method and modified star-shaped junction microfluidic device *(fig. 1)*. Liposomes are formed when three unique phases form a correct interface (OA phase, LO phase and IA phase). ***IA phase*** *(inner aqueous)* occupies the inner part of the liposome and contains an IVTT transcription/translation system, DNA, chaperones and salts needed for protein synthesis and integration into the membrane of the liposome. ***LO phase*** contains lipids (i.e. DOPC) and organic solvent (i.e. 2-octanol); it can also contain fluorescent lipids, such as Rh PE, for imaging. LO phase is critical for membrane bilayer formation. In OLA method, after the formation of the double emulsion, the excess of octanol and lipids dewets from the droplet and separates leaving double-layered liposomes. Octanol removal from liposomes is crucial as the correct bilayer cannot form in excess organic solvent. OA phase (outer aqueous) contains surfactants that help stabilize the droplets during initial synthesis and propagation along the microfluidic channels. An experiment is performed with a portable microfluidics station DropGen (*Droplet Genomics*).

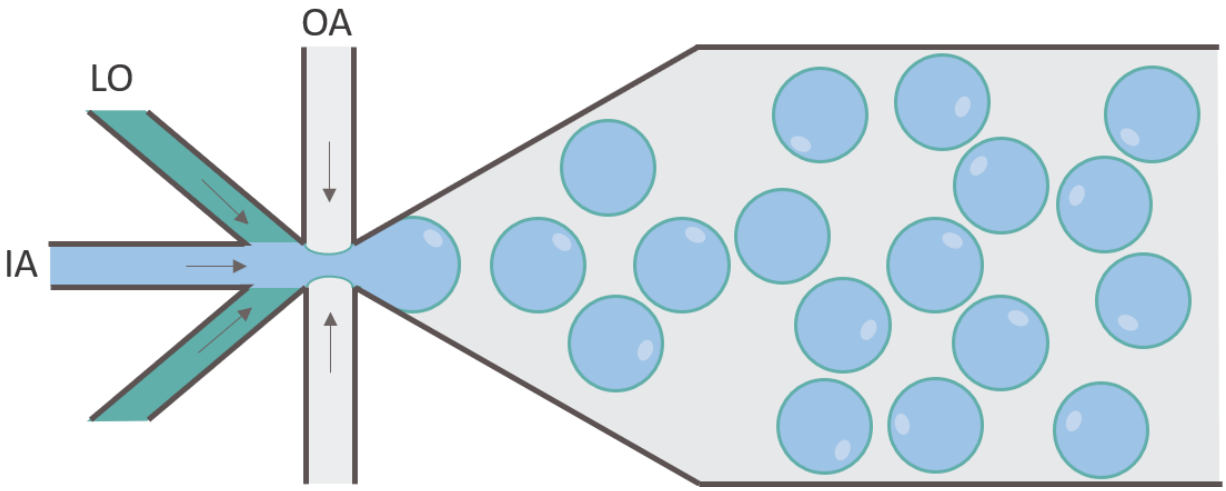

Fig. 1 A close-up of the interphase at the star-shaped junction of LipoDrop

EXPERIMENT PROTOCOL:

1. Solution preparations

LO solution: To prepare LO solution, lipids (100 mg/mL) and octanol are mixed in a 1:24 ratio, respectfully. Optionally, fluorescent lipids might be used for microscopy purposes: 1:1000 (labeled lipid: lipid). It is important to note, that a different variety of lipids can be used to form liposomes, depending on an experiment, although each configuration may vary in stability, permeability and other parameters. Most of our experiments were made with DOPC and Cholesterol.

OA solution: First, to prepare OA solution, one must make a buffer, that would be of similar composition to PURE*frex,* as it is important to keep similar osmolarity between the inside and the outside, and to provide some nutrients for the reaction (small molecules can permeate the lipid bilayer). The components of an outer solution buffer are listed in ***Tab 2***. Subsequently, ***Tab 3*** lists reagents needed for a complete OA solution.

IA solution: to make IA solution, PURE must be prepared as listed in the protocol. The final composition of IA solution is listed in **Tab. 4.**

	1X	2X	
Amino acids	0.3	0,6	mM
ATP	2	4	mM
GTP	2	4	mM
CTP	1	2	mM
UTP	1	2	mM
Spermidine	2	4	mM
Creatine phosphate	20	40	mM
DTT	1	2	mM
10-formyl-5,6,7,8-tetrahydrofolic acid	10	20	mg/mL
Potassium glutamate	100	200	mM
Mg(OAc)2	13	26	mM
HEPES-KOH (pH 7.6)	50	100	mM

Tab. 2 *Components for OA buffer*

	Volume %	For 1 mL solution
OA buffer 2x	50	500 µL
Glycerol	7	70 µL
2M Glucose	10	100 µL
Ethanol	10	100 µL
10% P188	23	230 µL

Tab. 3 *Components for OA solution*

	Volume %	For 300 µL solution
PURE buffer 2x	50	150 µL
Glycerol	7	21 µL
2M sacharose	10	30 µL
ddH2O	10	99 µL

Tab. 4 *Components for IA solution*

2. Syringe preparation

Fill the syringes with appropriate solutions: 1 mL plastic syringe for OA solution; 100 µL glass syringe for IA solution and 50 µL glass syringe for LO solution (use a pipette: gently touch filled pipette tip to the syringe opening and pull down the syringe grasp; make sure to avoid any bubbling). Secure on the needles and then the tubing. Carefully press the syringe grasp up until devoid of air. Place the syringes vertically in appropriate places on the pumping device. Make sure to secure them firmly on with all holders (3 separate ones). See ***Fig. 2*** for details.

Note*: the tubing should be appropriate length (enough to reach the channels but not wasteful).*
Note 2*: check the air syringes before every coating procedure: the plunger of the syringe should be pulled back.*
Note3: *Use tweezers when handling the tubing.*

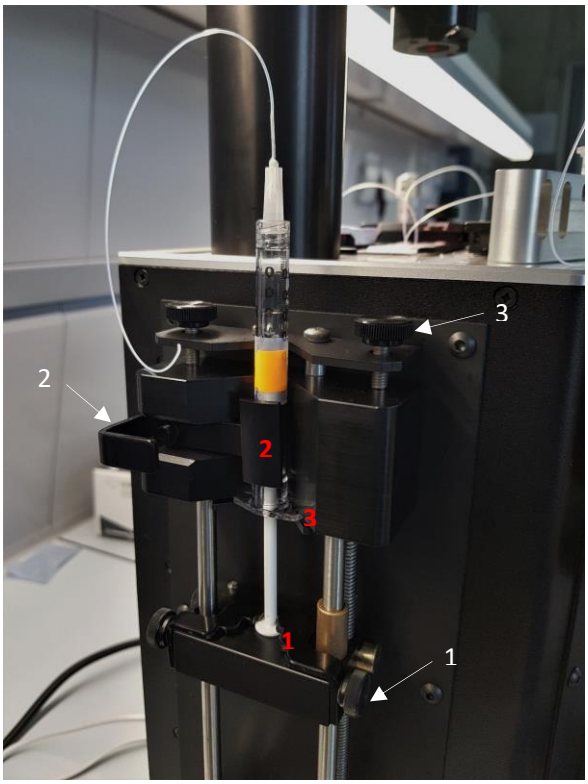

Fig. 2 Syringe placement setup on ***Dropgen*** portable microfluidic station.Red numbers indicate where the syringes should be secured, while white numbers mark appropriate screws for fastening the syringes in place.

3. Connecting the *DropGen* portable microfluidics station

a) Turn on the station by plugging it to the electrical socket. Simultaneously, turn on the computer and connect to DropGen Wi-Fi. Then visit the website: *192.168.1.100/instrument/dashboard*

b) For this experiment a default 3 syringe interface can be used. There is an option to name the pumps by selecting **Pump settings** in the **Settings** section found on the same page.

c) Camera view window should appear on the left part of the website (if not, restart the device or the webpage, or both).

d) To focus the camera, use the silver knob on the pumping device. For quick adjustment press ***Auto adjust*** on the webpage right below the camera view. It is possible to adjust Illumination, Exposure and FPS manually.

e) Pumping velocities can be regulated on the right panel of the website.

4. Syringe priming

To fill the tubes with material and make sure that syringes are firmly placed on the device **press purge** until you see the material visibly at the end of the tube. Make sure there are no bubbles in the tubing or in the syringes as they create instabilities of the system.

Before work, make sure that the pump device is clean from any dust as it potentially could clog the channels of devices. Prepare wipes for cleaning the waste (they should be right under the waste tubing).

5. Microfluidic device scheme

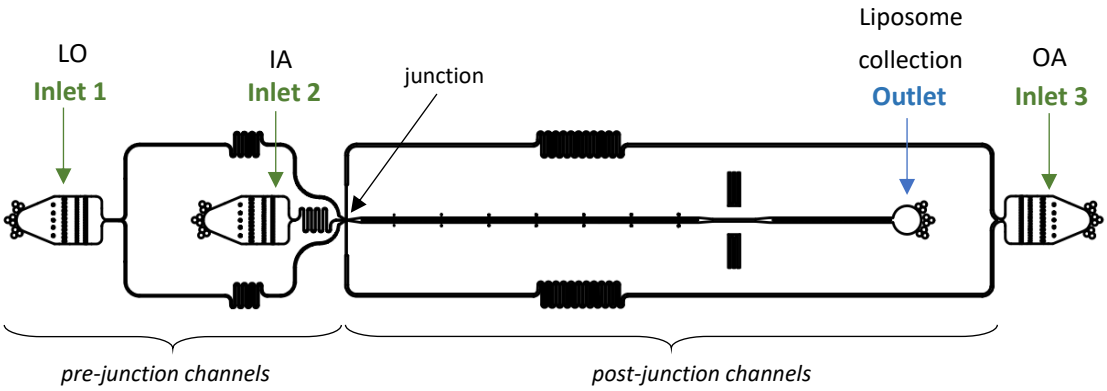

Fig. 3. Scheme of Lipodrop 2.0 microfluidic device. Green arrows indicate different inlets and correct configuration of the phases while blue arrow marks the outlet where the liposomes will be collected from.

6. Liposome synthesis

*! These steps for liposome synthesis are a only rough estimation for the process. One should consider that each microfluidic device is slightly different (channel height, proportions, etc.), some of which might have impurities or defects that would require adjustments to the suggested velocities. Keep in might that the process is dynamic and requires continuous attention. One of the most important parts of the process is achieving a correct and stable phase interface at the junction (See **Fig. 4**)!*

a) Put the tubing connected to the syringes to the appropriate inlets: LO to inlet 1, IA to inlet 2 and OA to inlet 3.
b) Insert the tubing to the outlet.
c) First, turn up the infusion rate of OA to 200 µL/h and observe the initial infusion. After the solution has entered the channels turn the infusion rate off.
d) Second, turn LO and IA infusion rates to 200 µL/h and turn them off immediately after they appear.
***Note:** OA should reach the post junction first, but only after LO and IA has appeared in the inlets. That ensures that excess air leaves the microchannels making easier to proceed in shaping the correct interphase.*
e) Wait for the OA to reach the junction on its own, and then turn on all of the phases.
f) Adjust the infusion rates like so: OA 200-400 µL/h; IA 100-300 µL/h and LO 150-400 µL/h. Slight differences in devices may result in an adjustment of flow rates of the solutions.
***Note:** These flow rates are not true values for flow rates as the syringes vary in volume. To extract real flow rates, divide IA flow rate values from 10.56, and LO from 22.07 (default settings are set for 1 mL syringes)*
g) The aim is to get a junction looking like ***Fig. 4.*** To reach it, adjust flow rates of the three phases.
h) After liposomes start to form correctly, change the outlet tube to a new one and start collecting the liposomes.
***Note:** The microtube that collects the liposomes should be kept on ice to prevent any synthesis before all liposomes are gathered.*
i) During a successful experiment, the liposome formation is highly stable, and can proceed up to several hours (or until one of the solutions has ran out).
***Note:** It is also possible to disconnect a tube, refill the syringe (draw the solution through the tube) and continue the experiment. It is suggested to change the tubing appropriately, to prevent the collection of the unnecessary waste.*
f) After the synthesis is done, draw tubing out of the inlets/outlets and turn off the flow rates.
***Note:** It is possible to reuse the device, but it should be washed through with a buffer.*
g) The collected liposomes should be incubated appropriately in 37 ℃ for 4-6 hours, depending on the experiment.

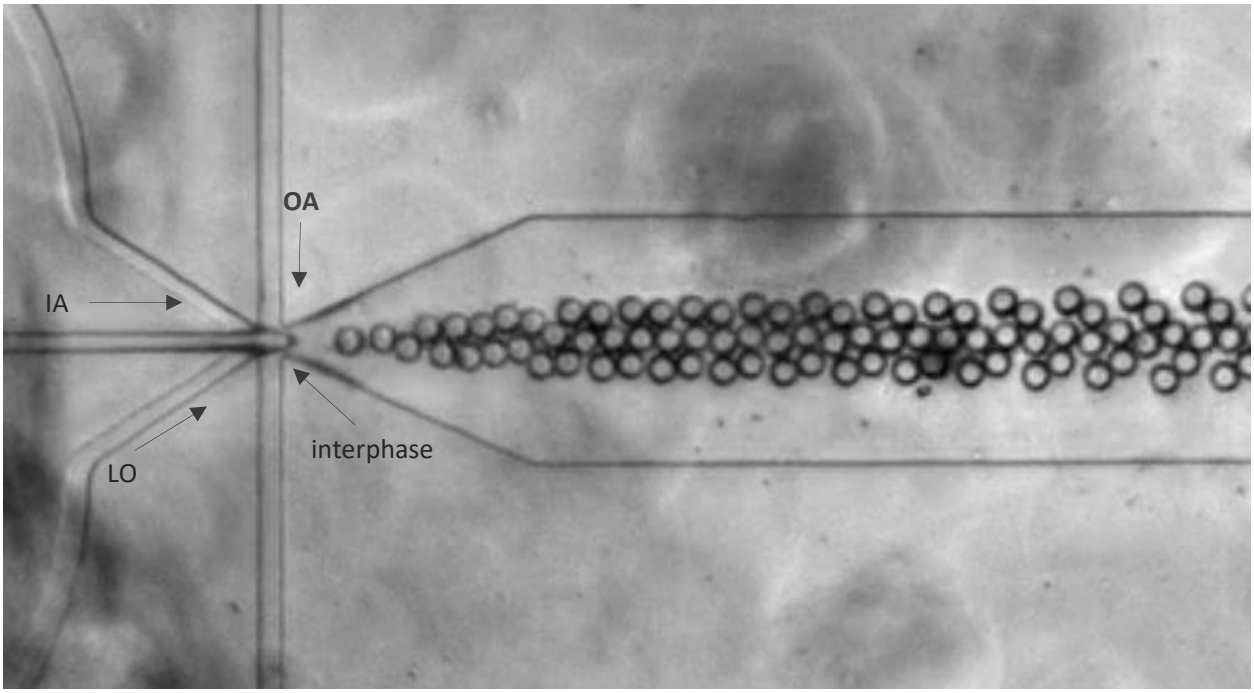

Fig. 4. A correct junction formation during liposome production

ADDITIONAL OBSERVATIONS AND IDEAS:

Possible mistakes & troubleshooting

- Make sure to check the correct arrangement of the microfluidic device before putting in the tubing.
- Check for the defects before every measurement to avoid wasting time and reagents on a defected device. Defects might include dust particles, closed channels, detached PDMS, discontinuous channels etc.
- Some clogging might happen due to small dust entering the microchannels. Sometimes physical pressure on the device (i.e. with tweezers) might unclog the microchannels.