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Protocol to engineer nanofilms embedded lipid nanoparticles for controlled and targeted drug delivery (NECTAR)

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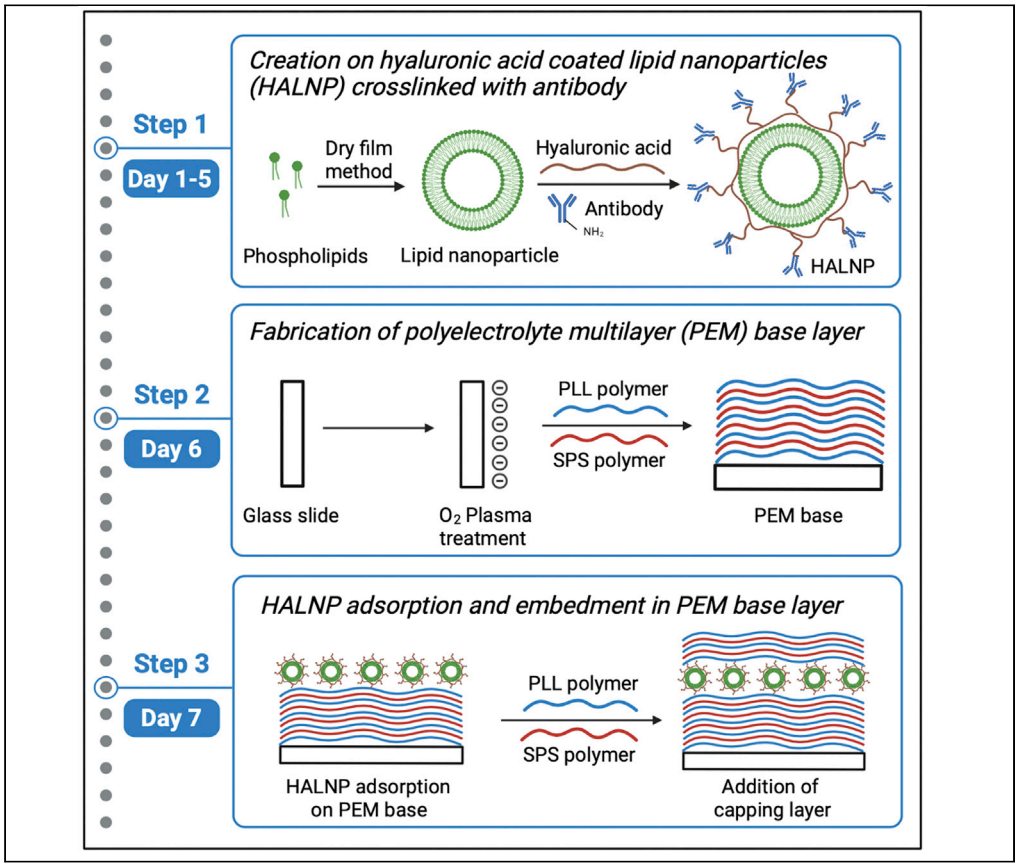
Protocol to engineer nanofilms embedded lipid nanoparticles for controlled and targeted drug delivery (NECTAR)



We present a protocol to engineer a substrate-mediated delivery platform comprising hyaluronic acid-coated lipid nanoparticles (HALNPs) embedded into polyelectrolyte multilayer (PEM) films. This platform allows controlled spatiotemporal release of lipid nanoparticles (LNP) by embedding them within the polyelectrolyte multilayer films matrix. HALNP conjugate with antibodies also adds the ability for targeted delivery. The use of LNP enables this platform to encapsulate both hydrophobic and hydrophilic drugs. This platform can easily be reproduced and utilized for various biomedical drug delivery applications.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

NECTAR platform for controlled spatiotemporal release of lipid nanoparticles (LNP)

Polyelectrolyte multilayer films embedded LNPs for controlling drug delivery

Hyaluronic acid-coated LNP (HALNP) conjugate with antibodies for targeted delivery

HALNP encapsulates both hydrophobic and hydrophilic drugs

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Protocol to engineer nanofilms embedded lipid nanoparticles for controlled and targeted drug delivery (NECTAR)

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SUMMARY

We present a protocol to engineer a substrate-mediated delivery platform comprising hyaluronic acid-coated lipid nanoparticles (HALNPs) embedded into polyelectrolyte multilayer (PEM) films. This platform allows controlled spatio-temporal release of lipid nanoparticles (LNP) by embedding them within the polyelectrolyte multilayer films matrix. HALNP conjugate with antibodies also adds the ability for targeted delivery. The use of LNP enables this platform to encapsulate both hydrophobic and hydrophilic drugs. This platform can easily be reproduced and utilized for various biomedical drug delivery applications.
For complete details on the use and execution of this protocol, please refer to Hayward et al. (2015, 2016a, 2016b), Hayward and Kidambi (2018), and Kidambi and Hayward (2022).

BEFORE YOU BEGIN

Buffer preparation

Timing: 2 h

1. Prepare PBS (phosphate buffered saline) buffer, HEPES (4-(2-Hydroxyethyl)piperazine-1-ethane-sulfonic acid) buffer, borate buffer, and sodium acetate buffer for HALNP synthesis experiment.

Note: It is advisable to prepare fresh buffers as per the recipe provided in the materials and equipment section. If using commercially available buffers, make sure to adjust their concentration and pH values to the desired amount as mentioned in this protocol. Pass these buffers through a 0.2 μm syringe filter before each experiment to make sure they are sterilized and free from contaminants for every batch of nanoparticles. The buffers can be autoclaved for additional sterilization after the preparation step, but it is not necessary as filter sterilization is enough.

2. Prepare PLL (Poly-L-Lysine hydrochloride) and SPS (Poly(sodium 4-styrene sulfonate) polymers for creating the PEM films.

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Note: During the experiment, if the used polymer solutions become cloudy, appropriately discard them, and use a fresh batch of the polymer solution. The polymer solutions usually start appearing cloudy after being used 10 times.

General lab preparation

Timing: 2–3 h

3. Rotary evaporator check:
   a. Examine whether the heating plate is working properly and that the temperature stabilizes to set values within ± 5°C.
   b. Check the functioning of the temperature sensor and rotor.
   c. Check if the vacuum pump is working properly and if any of the pipes connecting the pump to the rotor have any leaks.
4. Clean a 100 mL round bottom flask to be used for preparing liposomes by adding 20 mL of 100% pure ethanol to the flask and placing it in rotavapor for 30 min. Set temperature to 70°C and rotation speed to 60 rpm in the rotavapor equipment.

   Note: If using a round bottom flask with a different volume, change the volume of 100% pure ethanol accordingly. This step can be performed for more than 30 min, and the rotation speed can be modified as per the lab practices. It is best to set the temperature to 70°C or less as the boiling point of pure ethanol is 78°C.

5. Check ultracentrifuge.

   Note: The following steps are for Optima MAX-XP Ultracentrifuge to be used with the TLA-100.3 rotor. The manufacturer details of ultracentrifuge, rotor, and rotor tubes are mentioned in the key resources table.

   a. Ensure that the two O-rings in the lid of the TLA-100.3 rotor are intact. Inspect the rotor for any other kind of damage.
   b. Ensure that the ultracentrifuge chamber and rotor are dry. Wipe off any condensed droplets using an absorbent towel.
   c. Load the 3.5 mL tubes used with the TLA-100.3 rotor to about 80% capacity with the sample and place them symmetrically inside the rotor.
   d. Make sure that the lid is firmly tightened on the rotor before starting ultracentrifugation.
   e. Turn on the vacuum system and check if the reading goes below 10 microns. Press the start button only after the vacuum is achieved.
   f. Observe whether it attains the desired rotation speed of 135,000*g.

   Note: Further look into the instructions manual for using this ultracentrifuge. If using a different model of ultracentrifuge and rotor, make sure to take additional precautions as specified by the manufacturer.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Poly-L-Lysine (PLL) hydrochloride (M.W. 15,000–30,000)	MilliporeSigma	Cat# 26124-78-7
Poly(sodium 4-styrene sulfonate) (SPS) (M.W. 70,000)	MilliporeSigma	Cat# 25704-18-1

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Hyaluronic acid (HA)	MilliporeSigma	Cat# 53747
1, 2- Dipalmitoyl-sn-glycero-3-phopshoethanolamine (DPPE)	MilliporeSigma	Cat# P1348
Cholesterol (CHOL)	MilliporeSigma	Cat# C8667
1-ethyl-3-(3-dimethylaminopropyl) carbomiide (EDAC)	MilliporeSigma	Cat# 341006
L α-Phosphatidylcholine (PC)	MilliporeSigma	Cat# P3556
TopFluor cholesterol	Avanti Polar Lipids	Cat# 810255
Boric acid (H3BO3)	Sigma-Aldrich	Cat# B6768
Glacial acetic acid (CH3COOH)	Sigma-Aldrich	Cat# PHR1748
HEPES free acid (C8H18N2O4S)	Sigma-Aldrich	Cat# 391338
Sodium acetate (CH3COONa)	Sigma-Aldrich	Cat# S2889
Sodium chloride (NaCl)	Sigma-Aldrich	Cat# S9888
Potassium chloride (KCl)	Sigma-Aldrich	Cat# P3911
Sodium phosphate dibasic (Na2HPO4)	Sigma-Aldrich	Cat# 94046
Potassium phosphate monobasic (KH2PO4)	Sigma-Aldrich	Cat# P8709
MES (2-[morpholino]ethanesulfonic acid)	Sigma-Aldrich	Cat# M3671
Hydroxylamine-HCl	Thermo Fisher Scientific	Cat# 26103
Sulfo-NHS (N-hydroxysulfosuccinimide)	Thermo Fisher Scientific	Cat# 24510
100% Pure ethanol (200 Proof)	Decon Labs	Cat# 2701
Software and algorithms		
ImageJ	N/A	N/A
Photoshop	N/A	N/A
Biorender	N/A	N/A
Other		
Syringe	Sigma-Aldrich	Cat# Z116866-100EA
Syringe filter	Sigma-Aldrich	Cat# WHA10462200
Mini extruder apparatus	Avanti Polar Lipids	Cat# 610020
Polycarbonate membranes 0.8 μm	Avanti Polar Lipids	Cat# 610009
Polycarbonate membranes 0.4 μm	Avanti Polar Lipids	Cat# 610007
Polycarbonate membranes 0.2 μm	Avanti Polar Lipids	Cat# 610006
Polycarbonate membranes 0.08 μm	Avanti Polar Lipids	Cat# 610005
Filter supports	Avanti Polar Lipids	Cat# 610014
Heating plate (model: Isotemp)	Fisher Scientific	N/A
Ultracentrifuge (model: Optima Max-XP)	Beckman Coulter Life Sciences	Cat# 393315
Ultracentrifuge rotor (model: TLA-100.3)	Beckman Coulter Life Sciences	Cat# 349490
Ultracentrifuge tubes (3.5 mL tubes for TLA-100.3 rotor)	Beckman Coulter Life Sciences	Cat# 349622
Rotary evaporator (model: Rotavapor R-124)	BUCHI	Cat# R-124
Inverted microscope (model: Axiovert 40 CFL)	ZEISS	N/A
Microscope camera (model: ProgRes C3)	Jenoptik	N/A
Plasma cleaner (model: High Power Expanded Plasma Cleaner)	Harrick Plasma	Cat# PDC-001-HP
Ultrasonic bath (model: FS30D Ultrasonic Cleaner)	Fisher Scientific	N/A
Vortex (model: Maxi Mix II Vortex Mixer)	Fisher Scientific	Cat# NC0453190

MATERIALS AND EQUIPMENT

PLL solution

Reagent	Final concentration	Amount
PLL (MW~30 kDa, powder)	0.5 mg/mL	5 mg
NaCl (powder)	0.15 M	87.6 mg
Deionized (DI) Water	N/A	10 mL
Total	**N/A**	**10 mL**

Note: Store in 4°C refrigerator.

SPS solution		
Reagent	Final concentration	Amount
SPS (MW~70 kDa, powder)	30 μM	21 mg
NaCl (powder)	0.1 M	58.5 mg
Deionized (DI) Water	N/A	10 mL
Total	**N/A**	**10 mL**

Note: Store in 4°C refrigerator.

HEPES buffer		
Reagent	Final concentration	Amount
HEPES Free Acid (C8H18N2O4S)	20 mM	477 mg
Deionized (DI) Water	N/A	100 mL
Total	**N/A**	**100 mL**

Note: Adjust pH to 7.4 ± 0.05 and store at room temperature.

Borate buffer		
Reagent	Final concentration	Amount
Boric Acid	100 mM	618 mg
Deionized (DI) Water	N/A	100 mL
Total	**N/A**	**100 mL**

Note: Adjust pH to 8.6 ± 0.05 and store at room temperature.

Sodium acetate buffer		
Reagent	Final concentration	Amount
Sodium Acetate	100 mM	820 mg
Deionized (DI) Water	N/A	100 mL
Total	**N/A**	**100 mL**

Note: Adjust pH to 5.0 ± 0.05 and store at room temperature.

1× PBS buffer		
Reagent	Final concentration	Amount
NaCl	136.89 mM	8 g
KCl	2.68 mM	0.2 g
Na_2HPO_4	10.14 mM	1.44 g
KH_2PO_4	1.76 mM	0.24 g
Deionized (DI) Water	N/A	1,000 mL
Total	**N/A**	**1,000 mL**

Note: Adjust pH to 7.4 ± 0.05 and store at room temperature.

MES buffer		
Reagent	Final concentration	Amount
MES (2-[morpholino]ethanesulfonic acid)	0.1 M	195.24 mg
NaCl (powder)	0.5 M	292.2 mg
Deionized (DI) Water	N/A	10 mL
Total	**N/A**	**10 mL**

Note: Adjust pH to 6.0 ± 0.05 and store at room temperature.

EDAC solution in 0.1 M MES buffer		
Reagent	Final concentration	Amount
EDAC	0.4 M	310.48 mg
MES buffer	0.1 M	5 mL
Total	**N/A**	**5 mL**

Note: Adjust pH to 6.0 ± 0.05. Prepare fresh solution before the experiment.

Sulfo-NHS solution in 0.1 M MES buffer		
Reagent	Final concentration	Amount
Sulfo-NHS	0.4 M	434.26 mg
MES buffer	0.1 M	5 mL
Total	**N/A**	**5 mL**

Note: Adjust pH to 6.0 ± 0.05. Prepare fresh solution before the experiment.

Hydroxylamine-HCl solution		
Reagent	Final concentration	Amount
Hydroxylamine-HCl	1 M	694.9 mg
Deionized (DI) Water	N/A	10 mL
Total	**N/A**	**10 mL**

Note: Adjust pH to 8.5 ± 0.05. Prepare fresh solution before the experiment.

STEP-BY-STEP METHOD DETAILS

HALNP synthesis

Timing: 5 days

Figure 1 shows the schematic for the creation of Hyaluronic acid (HA) coated lipid nanoparticles (LNP) crosslinked with antibody for targeted delivery of therapeutic cargo. These liposomes can efficiently encapsulate both hydrophobic and hydrophilic compounds. HA has carboxylic acid groups (COOH) that impart an anionic charge at physiological pH. Thus, coating with HA mediates steric hindrance and provides prolonged stability to the liposomes in suspension. Further HA can be

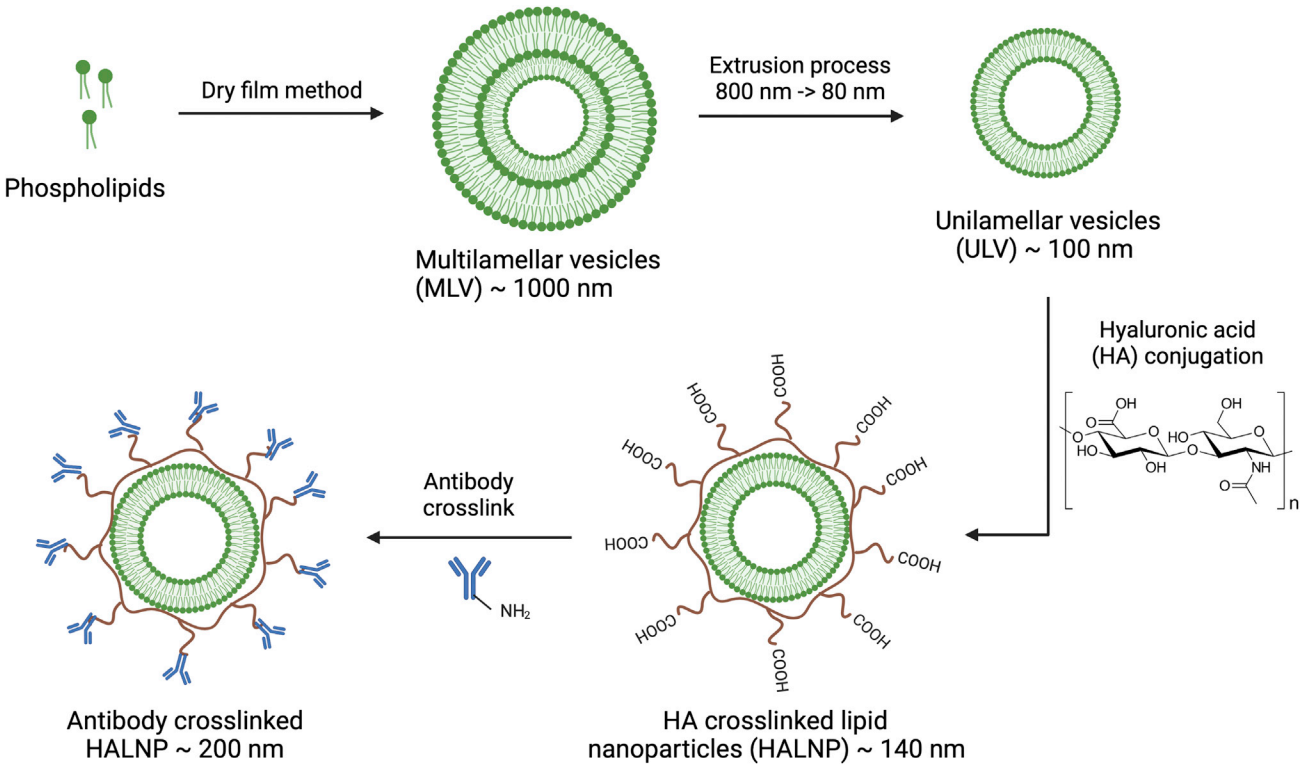

Figure 1. Schematic overview for creating hyaluronic acid-coated lipid nanoparticles (HALNP) crosslinked with antibody for targeted delivery

conjugated with various antibodies via amide coupling reaction to mediate tissue-specific targeted delivery.

1. Synthesize multilamellar vesicles (MLVs) (Day 1: 6 h).
   a. Add the three lipids PC (~ 25 mg), DPPE (~ 7.5 mg), and CHOL (~ 4.2 mg) in a 3:1:1 molar with a total lipid mass of nearly 37 mg in 100% pure ethanol in a 100 mL round bottom flask.

   Note: Lipid mass can be scaled up or down as required. The amounts of other reagents also need to be changed accordingly.

   b. For tracking purposes, the fluorescent tag can be added to HALNPs by adding 50 μg of TopFluor Cholesterol along with other lipids in the previous step.

   Note: The amount of TopFluor Cholesterol can be scaled in proportion to the amount of lipids used in the previous step.

   c. Mix them in a rotary evaporator at 65°C for 30 min at 60 rpm speed till a clear solution appears, indicating that the lipids have completely dissolved in ethanol.

   Note: Lipids can be mixed for a longer time until the solution appears clear.

   d. Turn on the vacuum pump to evaporate ethanol and form a thin film of lipids on the wall of the flask. Let it run for 1 h at 65°C (~350 mbar) followed by another 30 min under a complete vacuum (<100 mbar). Reduce the rotation speed of rotavapor to 30 rpm in this step to obtain a uniform thin film of lipids onto the flask.

e. Pass an indirect stream of nitrogen gas over the top of the flask using a nitrogen gun for 10 min to remove the traces of remaining ethanol.

Note: The time for this step can be modified depending on the removal of all the ethanol, leaving behind a dry lipid film on the flask. If the nitrogen gun is unavailable, install a pressure regulator and a shutoff valve on one end of the pipe connected with the nitrogen cylinder. Pass the nitrogen gas stream from the other end of the pipe connection directly over the flask. Carefully monitor the outlet pressure of nitrogen gas from the pipe and maintain it below 5 psi during this step. To enhance safety and ease of use, it is recommended to perform this step using a nitrogen gun.

f. Hydrate the dry lipid film with 20 mM HEPES buffer to a final lipid concentration of 10 mg/mL.

Note: Alternatively, 1× PBS buffer can be used to rehydrate the lipid film.

g. Vortex the solution for 10 min at the highest intensity of the device and let it sit overnight in a 4°C refrigerator.

2. Reduce lipid nanoparticle size and prepare HA solution (Day 2: 6 h).
   a. Place the mini extruder apparatus over the heating plate and set the temperature to 65 ± 3°C.
   b. Vortex the MLV solution for 10 min at the highest intensity of the device before starting the extrusion process.
   c. Pass 1 mL at a time of MLV solution through the extruder apparatus containing polycarbonate membranes. The steps to assemble the extruder apparatus with polycarbonate membranes and filter supports are illustrated in Figure 2.
   d. Conduct the extrusion process in stepwise manner of decreasing pore size of the polycarbonate membranes (800 nm -> 400 nm -> 200 nm -> 80 nm).

Note: Use an additional filter support (two in total) for each internal membrane support while extruding the particles through 200 nm and 80 nm membranes.

e. Pass each mL of the MLV solution through a polycarbonate membrane 21 times. Switch to the next membrane in the order of decreasing pore size (as mentioned in previous step k) and then pass the solution through that membrane 21 times.

Note: The MLV solution usually achieves a homogeneous single-particle distribution when passed through a membrane 21 times. Users can perform this step more or a lesser number of times as per their experiments.

   f. Store the LNP solution overnight in a 4°C refrigerator before starting the HA crosslinking step.
   g. Prepare HA solution with a concentration of 2 mg/mL by adding 20 mg of 1.65 MDa HA to 10 mL of sodium acetate buffer in a 15 mL Falcon tube. Incubate the HA solution overnight on a shaker table at 60 rpm speed at room temperature.

3. HA surface functionalization (Day 3: 10–12 h).
   a. In a 20 mL glass vial, combine 2 mL of HA solution (4 mg of HA) with 120 mg EDAC (1–30 mass ratio of HA to EDAC) for HA activation. Place a magnetic stir bar of appropriate size inside the vial, adjust the pH of the solution to 4.0 and incubate for 2 h at 37°C on a stir plate with a 60 rpm stir speed.

Note: The amount of HA and EDAC can be scaled up or down in proportion to the lipid mass used for the preparation of MLVs. Start pH adjustment only after the EDAC salt has completely dissolved in the HA solution, indicated by the appearance of a clear solution.

   b. Purify the extruded LNP solution via ultracentrifugation (135,000 g, 1.5 h, 4°C) and resuspend in 1 mL of borate buffer (pH 8.6).

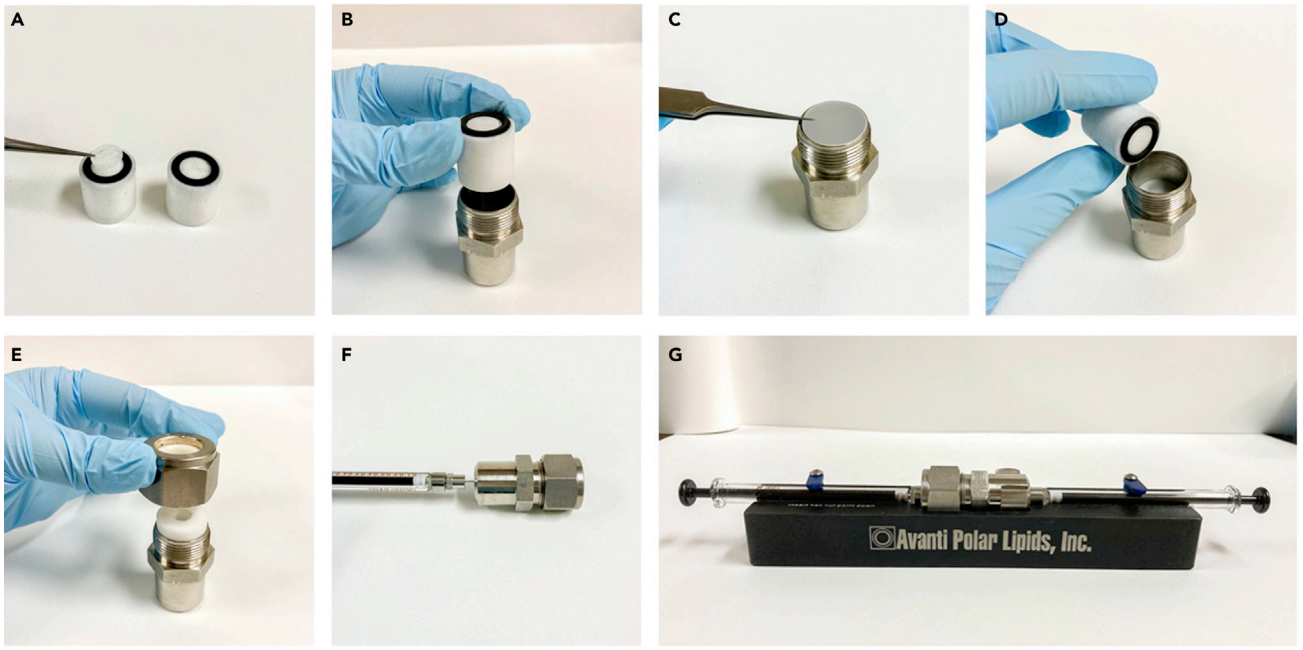

Figure 2. Steps for assembling the extrusion apparatus

Pre-wet the extrusion apparatus with DI water.

(A-G) (A) Place one filter support pre-wetted with DI water at the center of each Internal Membrane Support, (B) Insert one of the Internal Membrane Support inside the Extruder Outer Casing, (C) Place one Polycarbonate Membrane on top of the filter support inside the Extruder Outer Casing, (D) Place the second Internal Membrane Support over the polycarbonate membrane inside Extruder Outer Casing with its O-ring facing downward, (E) Place the Retainer Nut on the threaded end of the Extruder Outer Casing and tighten it, (F) Connect the needle of the glass syringe to one end of the extruder, (G) Connect another syringe to the extruder, put the apparatus on the heating block and place the swing-arm clips on top of both the syringes.

c. Combine the purified LNPs with activated HA solution in the glass vial, adjust the pH to 7.4, and incubate for 3 h at 37°C at 60 rpm stir speed on the stir plate.
d. After the 3 h of incubation as mentioned in the previous step, adjust the pH to 8.4–8.8 and incubate overnight at 37°C at 60 rpm stir speed on a stir plate.

4. Purify HALNPs (Day 4: 7 h).
   a. Purify the HALNPs three to four times via ultracentrifugation at 135,000 g for 1.5 h at 4°C.
   b. Wash with 3 mL of 1× PBS buffer after each run. Store the HALNPs in 3 mL of 1× PBS buffer at 4°C.

   Note: Storage volume of HALNPs in 1× PBS buffer can be changed as per the required HALNP concentration.

5. HALNP conjugation with antibody for targeted delivery (Day 5: 11 h).

EDAC/Sulfo-NHS carbodiimide crosslinking reaction is utilized for crosslinking carboxyl (COOH) group in HALNP to amine (NH_2) group in antibody.

a. Prepare 0.4 M EDAC solution and 0.4 M sulfo-NHS solution using 0.1 M MES buffer for both the solutions.
b. Purify 4 mg of HALNP via ultracentrifugation (135,000 g, 1.5 h, 4°C), and resuspend them in 100 μL of 0.1 M MES buffer.

Note: The amount of HALNP can be scaled up or down as per the experiment. The amounts of other reagents need to be changed accordingly.

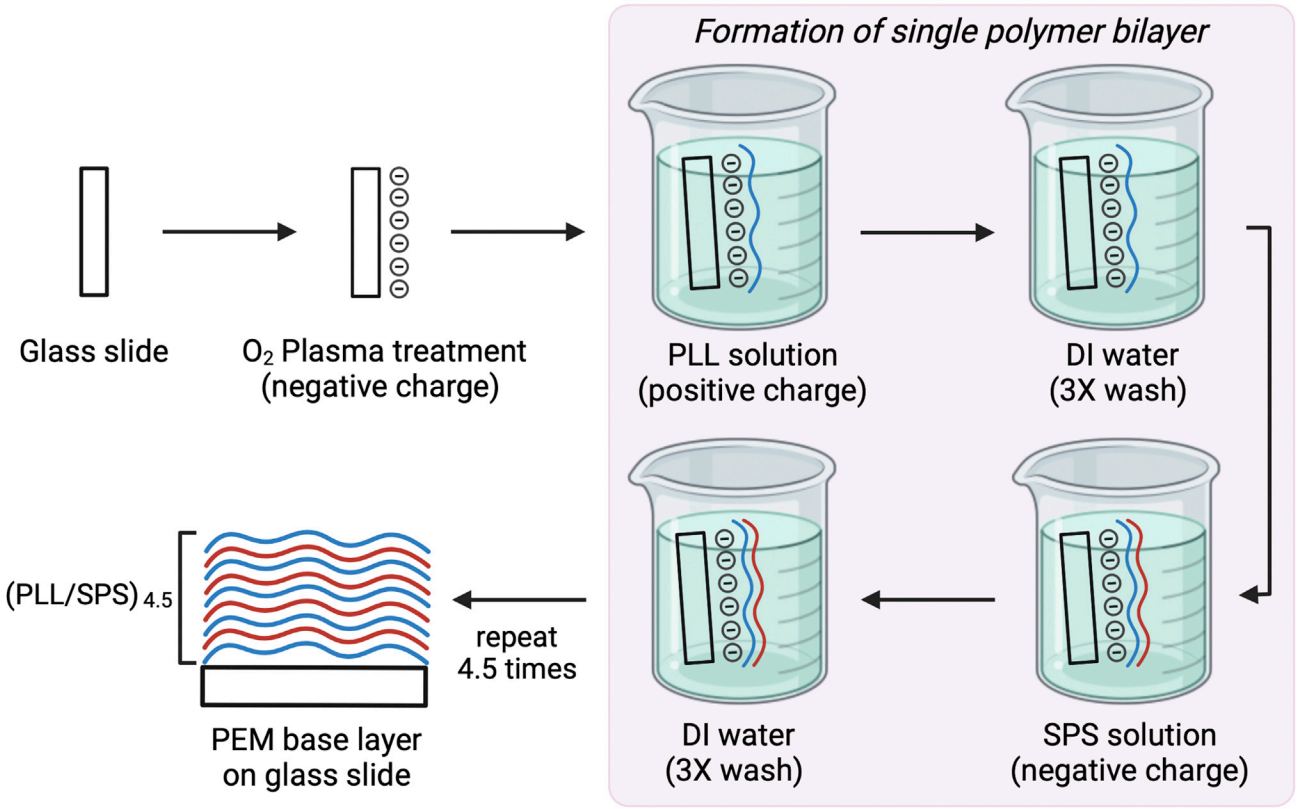

Figure 3. Schematic for fabrication of PEM base layer via layer-by-layer assembly of PLL and SPS polymer solutions

c. Add 100 μL of the 4 mg HALNP solution to 500 μL of 0.4 M EDAC solution in a glass vial or a falcon tube. Mix well and then add 500 μL of 0.4 M sulfo-NHS solution to the reaction vessel. Adjust the pH of the reaction mixture to 5.5–6 and incubate for 30 min on a shaker table at 60 rpm speed at room temperature.
d. Purify the reaction mixture via ultracentrifugation (135,000 g, 1.5 h, 4°C) and resuspend the HALNPs in 1 mL of 1× PBS buffer.
e. Add 10 μg of antibody to 1 mL of the HALNP solution in 1× PBS buffer and adjust the pH to 7.4. Incubate the solution for 3 h on a shaker table at 60 rpm speed at room temperature.

Note: A different amount of antibody can be used as per the experimental requirement.

f. Add 20 μL of 1 M hydroxylamine HCl solution (pH 8.5) to block the reactive residues. Stir for 15 min on a shaker table at room temperature at 60 rpm speed.

Polyelectrolyte multilayer base fabrication

Timing: 7 h (Day 6)

PEMs construction through the layer-by-layer assembly is a very versatile and reproducible method to fabricate highly ordered polymeric thin films on a material. They have been successfully utilized for substrate-mediated delivery of nano-drug carriers such as liposomes to achieve a sustained and controlled drug release system. In this study, the PEM base layer is utilized to immobilize HALNPs on PEM films and prevent the early release of the drug-loaded HALNPs. The steps for the preparation of the polyelectrolyte multilayer (PEM) base layer are illustrated in Figure 3.

6. Treat the glass slide with oxygen plasma using the plasma cleaner equipment to induce a negative surface charge.

   Note: A cell culture dish can also be used instead of a glass slide to prepare the PEM base layer.

   a. Turn the three-way valve on the front of the door to the right to bleed the chamber. Once the hissing sound of air stops, turn the three-way valve back to the vertical position.
   b. Open the door, place the glass slide in the chamber, shut the door and turn on the vacuum pump. Evacuate the chamber until the pressure is stable at around 100 mTorr.

   Note: While the chamber is evacuating, make sure that the valve of the oxygen tank is open, and the output pressure of the oxygen is stable at 10 psi.

   c. Turn the three-way valve to the left to bleed the Oxygen gas into the chamber.
   d. Wait until the pressure stabilizes between 400 mTorr and 450 mTorr.

   Note: Make sure that the reading on the input flow meter on the PlasmaFlo is 10 mm. If not, turn the knob below the input flow meter to adjust it to 10 mm.

   e. Turn on the RF power switch and wait for plasma to form. It usually takes about 10 s for the blue light to appear which is indicative of the plasma formation.
   f. Turn on the timer and leave it for 8 min to obtain plasma coating on the slide.
   g. After 8 min, turn off the RF power. Turn the three-way valve back to its vertical position and allow the chamber to evacuate to 150 mTorr.
   h. Once the chamber evacuates to 150 mTorr, turn off the pump.
   i. After the pressure reaches 1500 mTorr, bleed the chamber by turning the three-way valve to the right until you hear the hiss from the air.
   j. When the pressure reading is above 1800 mTorr, turn the three-way valve back to its vertical position.
   k. Open the door and retrieve the glass slide.

   Note: These steps for oxygen plasma treatment are for High Power Expanded Plasma Cleaner from Harrick Plasma (refer to key resources table). If using a different model of plasma cleaner, it is advisable to check its instruction manual and perform the experiment accordingly.

7. Coat the negatively charged glass slide with positively charged PLL solution by vertically dipping the glass slide into a beaker containing the PLL solution. Incubate for 20 min at room temperature.
8. After coating with PLL solution, wash the glass slide with DI water three times to remove the excess polymer. For each wash, place the glass slide vertically inside a DI water containing beaker and incubate for 5 min.

   Note: Use three different DI water containing beakers for each wash step.

9. Next, incubate the glass slide with negatively charged SPS solution by vertically dipping the glass slide into a beaker containing the SPS solution. Incubate for 20 min at room temperature. This would create a single PEM bilayer of PLL and SPS polymers.
10. Wash the glass slide with DI water (as described in step 8) three times for 5 min per wash to remove the excess polymer.

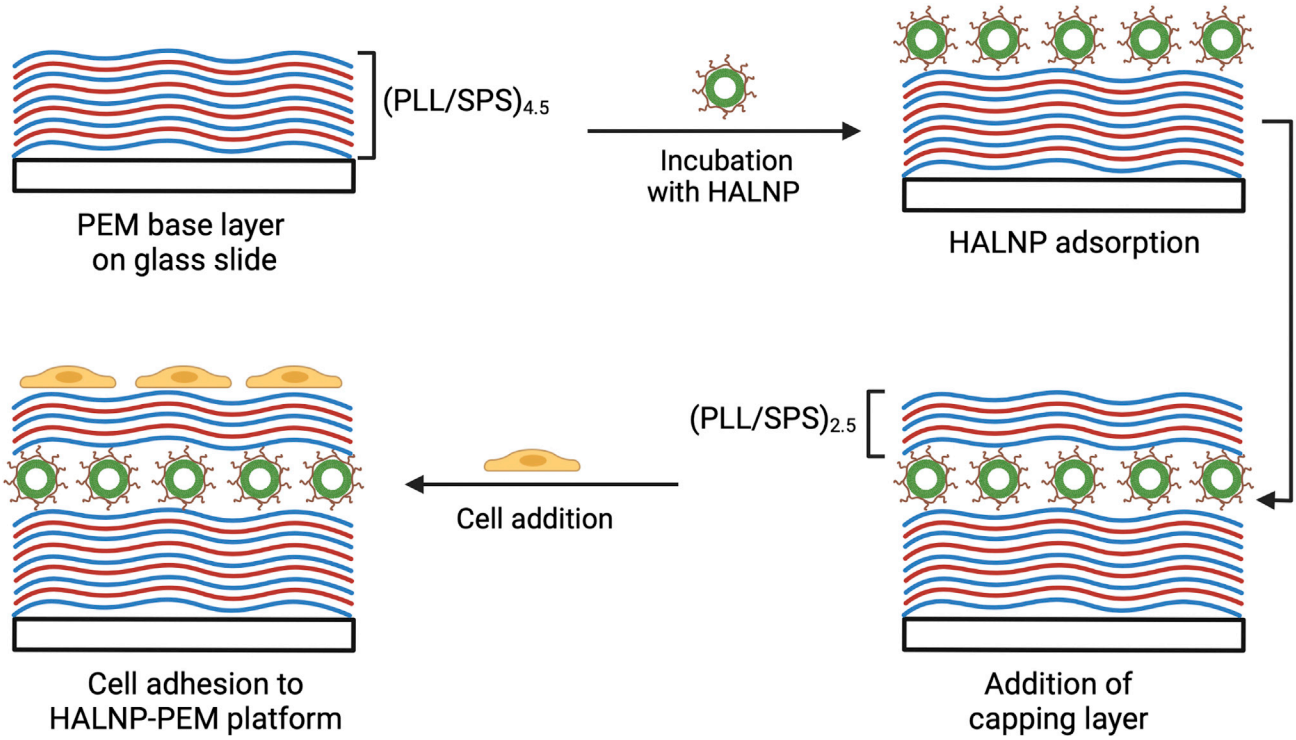

Figure 4. Schematic for nanoparticle absorption and embedment in PEM platform followed by cell adhesion

11. Repeat steps 7–10 three more times to obtain a total of four PEM bilayers of PLL and SPS polymers. Then perform steps 7 and 8 one more time to obtain 4.5 PEM bilayers of PEM ($(PLL/SPS)_{4.5}$) where PLL forms the topmost layer.

Note: Perform all the coating steps on a clean bench to ensure sterile conditions.

HALNP-PEM platform fabrication

Timing: 7 h (Day 7)

Figure 4 illustrates the steps for nanoparticle embedment in the PEM platform. HALNPs are immobilized on the PEM base layer by covalent interaction of negatively charged HALNPs with positive charged PLL polymer. The capping layer ($(PLL/SPS)_{2.5}$) on top of the HALNP-PEM system provides a highly controlled and sustained release profile of HALNPs without an initial burst release as observed in the early stages of the uncapped system (Figure 5E).

12. Incubate the PEM base layer ($(PLL/SPS)_{4.5}$) as prepared in step 11 with the HALNP solution.

Note: Use a concentration of 0.18 mg HALNP per cm^2 area of the glass slide or a cell culture dish.

Note: This step can be performed with a different concentration of HALNP as per the experiment.

13. Perform the HALNP incubation with the PEM base platform for 3 h at room temperature.
14. Following incubation, wash the surface three times with 20 mM HEPES buffer. Visualize the glass slide under Axiovert 40 CFL Zeiss inverted microscope with a fluorescent filter (absorbance: 495 nm, emission: 507 nm) and capture the images with the attached ProgRes C3 Jenoptik camera.

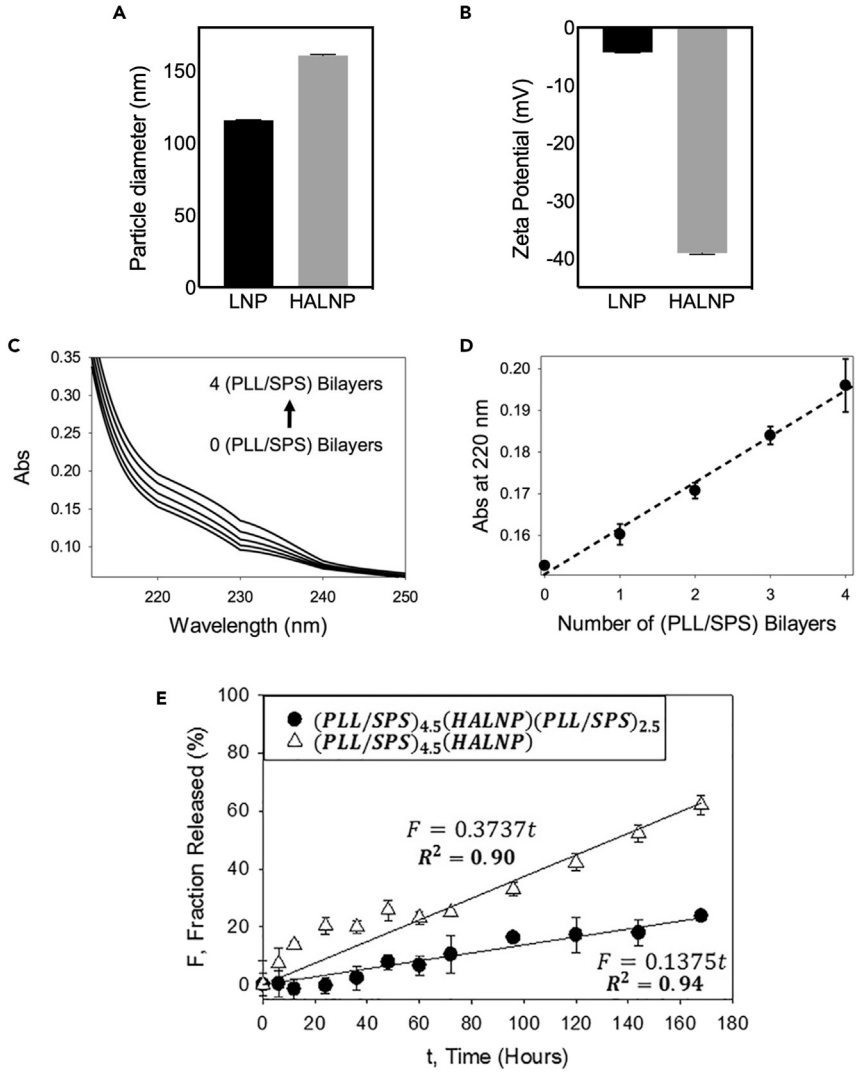

Figure 5. Characterization of HALNP-PEM assembly

(A) Dynamic light scattering analysis of LNP and HALNP.

(B) Zeta Potential analysis of LNP and HALNP.

(C and D) UV Absorbance Readings at 220 nm (characteristic for SPS) as a function of bilayer addition.

(E) HALNP release kinetics analysis from the non-capped [$(PLL/SPS)_{4.5}(HALNP)$] and capped [$(PLL/SPS)_{4.5}(HALNP)(PLL/SPS)_{2.5}$] HALNP-PEM platforms. Data are represented as mean ± SEM.

15. Following deposition of the HALNPs, add a capping layer of 2.5 bilayers of PLL and SPS polymers (two bilayers of PLL/SPS polymers and another layer of PLL polymer on top - $(PLL/SPS)_{2.5}$) on top of the HALNP layer. For coating PLL and SPS polymers, follow the steps from 7 to 10 as described under the 'polyelectrolyte multilayer base fabrication' section.

Note: Add the capping layer sequentially by hand on a clean bench to ensure sterile conditions.

EXPECTED OUTCOMES

In this protocol, we described a novel method to engineer a substrate-mediated delivery platform for the controlled and targeted release of lipid nanoparticles. This model would provide a

biocompatible and easily reproducible platform for a controlled temporal and spatial release of a variety of drugs.

Figure 1 provides a schematic overview for creating hyaluronic acid coated lipid nanoparticles (HALNP) crosslinked with antibody for targeted delivery. HALNP are created by self-assembly of phospholipids into multilamellar vesicles (MLV) which are then subsequently extruded through polycarbonate membranes (Figure 2) to create unilamellar vesicles of nearly 100 nm diameter. The particle surface is then modified using hyaluronic acid (HA) which is a polymer with hydroxy and carboxylic functional groups. This kind of design offers high biocompatibility, the capability to encapsulate both hydrophobic and hydrophilic drugs, and tunability for controlled drug release. HA provides stability to the particles and prevents them from aggregating in solution and the carboxy group in HA can potentially be crosslinked with the amine group in antibodies for tissue-specific targeting. Upon characterization of lipid nanoparticles through the dynamic light scattering (DLS) method, the particle size increases from 100 nm to nearly 150 nm upon HA crosslinking (Figure 5A). The zeta potential measurement shows a reduction in surface charge of nanoparticles from -4 mV to -38 mV upon HA crosslinking (Figure 5B) owing to the negative surface charge imparted by the carboxyl group of HA.

Figure 3 describes the fabrication method for the PEM base platform via layer-by-layer assembly of PLL (positively charged) and SPS (negatively charged) polymer solutions. The glass substrate is treated with plasma to impart a negative surface charge followed by the deposition of four bilayers of PLL/SPS and then another layer of PLL on top (4.5 bilayers) to achieve a uniform coating. The subsequent deposition of the PLL/SPS films is determined via UV-Vis spectrophotometry by measuring the characteristic absorbance values of SPS at 220 nm. The multilayered PLL/SPS coatings show a linear increase in UV-absorbance spectrum with the addition of every successive double-layer, thus validating the formation of PEM films (Figures 5C and 5D).

Figure 4 shows a step-by-step method to engineer the liposome-embedded PEM films. The negatively charged HALNPs are adsorbed on top of the positively charged PLL layer of $(PLL/SPS)_{4.5}$ bilayer assembly on the PEM substrate. A capping layer comprising 2.5 bilayers of PLL/SPS is added on top of the HALNP layer to immobilize the particles and impede their migration within the PEM sandwich.

The release kinetics analysis of HALNP from the non-capped $[(PLL/SPS)_{4.5}(HALNP)]$ and capped $[(PLL/SPS)_{4.5}(HALNP)(PLL/SPS)_{2.5}]$ HALNP-PEM platforms show that the capped HALNP-PEM system facilitates a linear release profile without an initial burst release as observed in the early stages of the uncapped system (Figure 5E). Thus, a capping layer immobilizes HALNPs on PEM films and provides a highly controlled and sustained nanoparticle release profile.

Lipid nanoparticle based drug delivery system has been of great interest for biomedical applications owing to several advantages such as high biocompatibility, tunability, controlled release, high drug loading capacity, reduced side effects, etc. Liposomes are often surface-functionalized with stealth polymers such as poly(ethylene glycol) (PEG) to enhance their circulation time and stability in *in-vivo* applications. However, PEGylation may have some detrimental effects and can induce immunogenic responses in the body. Hyaluronic acid (HA) utilized for coating lipid nanoparticles in this study offers several advantages over other conventional methods. HA is naturally produced in the body, thus HA coating circumvents the toxic effects and provides excellent biocompatibility to this design of LNPs. HA is also a natural ligand for CD44 surface receptor which is highly expressed in many forms of cancer, thus HALNP can directly be utilized for targeting cancer cells. The carboxyl group (COOH) in HA provides high negative charge density and stability to LNPs and can be conjugated with the amine group in various antibodies for targeted delivery. HALNP design also provides the flexibility to conjugate multiple ligands at a time and yield various functionalities to the system, thus providing an advantage over other nanoparticle-based delivery methods. Substrate-mediated liposome delivery

through PEMs is a promising approach for delivering numerous therapeutic compounds. However, current research on liposome-PEM systems is limited due to poor immobilization of liposomes onto PEMs which hinders the loading capacity and kinetics of drug release. In the NECTAR platform, the capping layer on top of the HALNP layer was employed to enhance nanoparticle immobilization, overcome burst kinetics, and provide a sustained release profile for HALNP along with the encapsulated therapeutic drug. We believe that this design can potentially be employed for the development of substrate-mediated delivery platforms for an array of disease models.

QUANTIFICATION AND STATISTICAL ANALYSIS

The difference between experimental groups was analyzed by one-way analysis of variance (ANOVA) and by a subsequent Turkey's multiple comparison test in Sigma Plot Software. For statistical analysis of all data, $p < 0.05$ was regarded as the lowest acceptable threshold for significance.

LIMITATIONS

This protocol describes a robust method to create substrate mediated delivery platform comprising lipid nanoparticles, however, the process for fabrication of this platform is labor-intensive. Manually creating the lipid nanoparticles and PEM substrate may cause batch-to-batch variability and thus the process needs to be optimized for macroscale production. The NECTAR platform can be very well utilized for controlled administration of therapeutics in adherent and suspension cell lines in an *in vitro* model, however current design limits its usage in *in vivo* applications. For pharmaceutical translation, this platform requires further modifications and can potentially be developed into an implantable device to enhance the local delivery of the therapeutics. Additionally, coating plates by hand can increase the chances of human error, and failure to carefully follow the temperature and pH sensitive steps can lead to the ineffective synthesis of the platform.

TROUBLESHOOTING

Problem 1

Lipid film can get a bit sticky with round bottom flask and won't come out easily (Section 'HALNP synthesis', step 1. f).

Potential solution

Vortex the part of the round bottom flask where lipids are stuck. This helps with getting lipids back into the HEPES solution. If vortexing doesn't work well, an ultrasonic bath (set to 42 kHz frequency) can be used for 5 min to detach the lipids from the flask.

Note: If the lipids are still sticking to the flask, ultrasonication can be done for additional 5 min.

Problem 2

Loss or disruption of lipids during the purification step (Section 'HALNP synthesis', step 3. b, step 4 a, and steps 5. b, d).

Potential solution

During washing steps, the HALNP pellet might start loosening and going back into the solution if left for over 10 min after finishing the rotation cycle. Also, it's important to be careful that the pipette tip doesn't touch the HALNP pellet while rehydrating after the washing step.

Problem 3

Difficulty in pushing lipid nanoparticles in the syringe through the polycarbonate membranes during the extrusion process (Section 'HALNP synthesis', steps 2. c, d, and f).

Potential solution

Sometimes users might experience that a lot of force is required to push lipid nanoparticles in the syringe through the polycarbonate membrane in the extrusion apparatus. This can particularly

happen while using membranes of smaller pore sizes (200 nm and 80 nm). In this case, try not pushing too hard as this might break the membrane inside the apparatus. Instead, let the nanoparticles contained in the syringe in the apparatus sit on the heating plate for a couple of minutes and try pushing it gently through the membrane again. If it still requires a lot of force, then it is possible that the particles weren't extruded properly through the previous larger pore-sized membrane. So, repeat the extrusion process through the previous larger pore-sized membrane in the sequence and then try again with this smaller pore-sized membrane.

Problem 4

Plasma cleaner chamber shows pink or colorless gas instead of light blue gas upon bleeding oxygen gas into the chamber (Section 'polyelectrolyte multilayer base fabrication', step 6. e).

Potential solution

Pure oxygen gas emits a light blue color upon excitation. The color shift to pink indicates the inclusion of Nitrogen and Hydrogen inside the chamber due to the presence of humidity. In such a case, first check the connections between the plasma cleaner and the oxygen cylinder to ensure that the settings are correct. Try to get rid of any moisture inside the chamber, then evacuate the chamber and let the pressure stabilize to about 100 mTorr. Bleed the oxygen gas into the chamber and check if the light blue color of oxygen plasma is observed.

Problem 5

Low fluorescence observed while visualizing HALNPs attached to the glass slide under the microscope (Section 'HALNP-PEM platform fabrication', step 14).

Potential solution

If fluorescent HALNP attached to the glass slide can't be visualized properly under the microscope, a few things can be tried for troubleshooting. Make sure the exposure time and florescent wavelength are set to correct values in the microscope. Try incubating the glass slide with HALNP for additional 3 h (or more if required) and check the slide under the microscope again. HALNP concentration can be increased in the incubation step. Revisit the coating steps to check for any errors and make sure that the PLL polymer (positive charge) was coated on the top of the slide before incubating with HALNPs.

RESOURCE AVAILABILITY

Lead contact

Requests for further information should be directed to and will be fulfilled by the lead contact, Srivatsan Kidambi (skidambi2@unl.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate or analyze datasets or code.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.L.H. and S.K.; Investigation, S.L.H.; Writing – Original Draft, R.P.; Writing – Reviewing & Editing, R.P. and S.K.; Funding Acquisition, S.K.; Supervision, S.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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