Protocol for Making DNA Microspheres – Large Batch

Last Updated 24 June 2014

Goal: Incorporate DNA into PLA microspheres. DNA serves as identification of specific tracer while PLA protects the DNA from degradation and damage. Uses Double Emulsion Solvent Evaporation Technique.

Outline:

Mix All reagents
↓
Create PLA Microspheres by Sonication
↓
Lyophilize PLA Microspheres for Storage.

Protocol Credit Christine Georgakakos (cbg46@cornell.edu)
Reference: Luo, D., Woodrow-Mumford, K., Belcheva, N., Saltzman, W.M. (1999) Controlled DNA Delivery Systems. Pharmaceutical Research, Vol. 16, No. 8 1300-1308.

Details:

I. Materials Required

a. Stock Materials (all in mirco lab RR B44)
   1. PLA - (polylactide) polymer 3001D (Nature Works)
   2. PVA – (Polyvinyl alcohol) 88% mol % hydrolyzed MW ~ 25,000 (Polysciences, Inc. #02975)
   3. Dichloromethane CH_2Cl_2
   4. DNA working solution
   5. Iron Oxide Powder
   6. Nuclease-free water
   7. Milli-Q water (upstairs, RR 158)

b. Tools
   1. Sonicator (Sonifier 150, Branson Ultrasonics Corp.) (Upstairs in RR 153)
   2. Freeze Dry System/ Freezone 4.5 (i.e. Lyophilizer) (Labconco) (Upstairs in RR 158
   3. Centrifuge capable of 4000 rpm (several around RR: rooms B72, B69A (works?), B52, B50(for last two, get permission before using))
   4. Centrifuge tubes
   5. Heating/stirring plate (generally found in fume hoods)
   6. Pippetes and pipette pump
   7. 125 ml flask
   8. 2 l flask or beaker
   9. Stir bars

c. Solutions
   1. 1% PVA Solution (50 ml)
      a. 0.5 g PVA

b. 40 ml dH_2O
c. Place flask on heating/stir plate at 37°C at max RPM overnight
d. Cover top of beaker/flask or solution will vaporize
e. When completely dissolved, add dH_2O to 50 ml
   ✯✯NOTE✯✯: PVA is difficult to dissolve, may want to dissolve over night. Will come out of solution if left for too long, but easier if make larger batches at a time

2. 0.3% PVA
   a. 3.0 g PVA
   b. 900 ml dH_2O
   c. Place flask on heating/stir plate at 37°C at max RPM
   d. Cover top of beaker/flask or solution will vaporize
   e. When completely dissolved and ready to use, add water to 1000 ml solution
      ✯✯NOTE✯✯: PVA is difficult to dissolve, may want to dissolve over night. Will come out of solution if left for too long.
3. Iron Oxide Solution
   a. Add 50 mg Iron Oxide to a small 1ml centrifuge tube
   b. Add 0.5 ml nuclease-free water to the 50 mg iron oxide tube. Shake well.
   c. This step can be done the day before to encourage dissolution of iron oxide into water.
4. DNA Solution
   a. Remove DNA Working Solution from freezer, flick/shake, centrifuge 6 sec, repeat till thawed
   b. Add 300 µl NUCLEASE-FREE water
   c. Shake, centrifuge solution to integrate DNA working solution with nuclease-free water.
      ✯✯NOTE✯✯: This should NOT be made ahead of time because DNA may degrade.

II. Procedure

✯✯NOTE✯✯: All steps involving Dichloromethane should be performed under a fume hood. This procedure is scaled to ~2.00 g PLA as starting material. At this volume, the sonicator (set to level 10) can effectively mix the entire volume. We have not experimented at scaling up at this level. In step a7 of the procedure, the procedure may be doubled to produce a larger batch.

a. <u>Double Emulsion Solvent Evaporation Technique</u>

1. In a 125 ml flask combine:
   ✯✯NOTE✯✯: If flask is too much larger than 125 ml, the sonicator will not effectively mix all completely and spheres will be too large.
   a. 2.0017 g PLA
   b. 20.02 ml Dichloromethane
2. Place solution on stir plate on high for ~1 hr until PLA is COMPLETELY dissolved.

3. Slowly add drop-wise 500 µl DNA solution and iron oxide solution to PLA-Dichloromethane solution while stirring (this new solution will be referred to as the polymer solution)
4. Have 0.3% PVA solution vigorously stirring during sonication
5. Sonication
    a. Materials: 2 L beaker or flask, large stir bar, 1000 ml 0.3% PVA solution, 40ml 1% PVA, ice bucket or beaker with ice ~ 1/3 full, DNA-PLA-Dichloromethane-iron oxide solution in a 125 ml flask, pipette pump, 2x10 ml pipettes or 2x40 ml pipettes, sonicator
    b. Sonicate polymer solution on ice for 15 s at level **10**. Repeat 3 times sloshing mixture between each repetition. Solution should become milky in appearance. (1st Emulsion)
    ✯✯NOTE✯✯: Bring tip of sonicator just under the surface of the solution. If sonicator is too deep, will not correctly emulsify.
    c. Slowly pipette 40 ml 1% PVA to 1st emulsion while stirring. There will be large visible spheres forming.
    d. Remove stir bar from mixture
    e. Sonicate mixture for 30 s, the remove and slosh contents to mix. Repeat 3 times. (2nd Emulsion)
    ✯✯NOTE✯✯: Bring sonicator tip just under surface of mixture. DO NOT bring tip all the way to the bottom of the flask near the glass. IF this is done spheres will form that are very large.
6. Slowly add 2nd Emulsion to 0.3% PVA using pipette
7. Continuously stir final solution (2nd emulsion + 0.3% PVA) for 3 hr at room temp.
8. Centrifugation
    a. Centrifuge final solution at 4000 rpm and 4°C for 10min
    b. Pour off liquid into hazardous waste container
    c. Wash microspheres with milli-Q water after have been centrifuged. Repeat step 8.
    d. Centrifuge for 5min, pour off water
9. Freeze microspheres in -80° freezer overnight
    ✯✯NOTE✯✯: Samples cannot be lyophilized unless they are entirely frozen
10. Place microspheres (in 50ml centrifuge tubes) into lyophilizer for 24 hr
11. Store in freezer in dry form

Making Substock and Working Solutions from Stock Solution

Key: Stock – S
Substock – SS
Working – W

Making Stock solution:
Stock solution is made from the dry DNA that is received from IDTA. The concentration of this solution should be 0.0001 M, or 100 µM. The exact amount of water added from the dry to S solution varies for each shipment. This is the case for the forward and reverse primers also. The dilution should take the number of Anhydrous nanomoles and add 10x that number of µL.
See figure at end for example numbers.

Making Substock Solution:
Dilute the S solution by a factor of 10. Concentraion of this solution should be 0.00001M.
Example: 5 µL stock solution added to 45 µL of nuclease free water.

Making Working Solution:
Dilute SS solution by factor of 10. Concentration of this solution should be 0.000001M with the total number of DNA copies in the solution being 6.02E17/L. Working solution for botht he DNA tracer and the forward and reverse primers are used in W solution concentrations. Make W solutions in 200 µL allocations for easier use in Tracer making.

✯✯NOTES✯✯:
Generally do not freeze and thaw samples too many times. Freezing and thawing degrades the DNA more quickly. For this reason, we make many working solutions at once, to minimize freezing and thawing of any one sample. The same is true for the SyberGreen used in qPCR. Divide the solution so that it can be used almost entirely in one experiment. When making many batches of microspheres, the limiting step is centrifugation. Then it is useful to use several centrifuges at once.