



2013 GEM⁴ BioNanotechnology Summer Institute

Cancer Nanotechnology and Cellular Mechanics

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University of Illinois at Urbana-Champaign

THERAPEUTIC NANOTECHNOLOGY LAB MODULE

Location: BioNano Lab, 3119 Micro and Nanotechnology Laboratory (MNTL)

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Purpose and Expected outcome:

The purpose of this laboratory module is to provide an introduction and a hands-on demonstration of the formulation and evaluation of drug delivery nanoparticles (NPs) for cancer therapy. Students will be able to prepare nanoparticles that contain anti-cancer drugs internally and a ligand coated on NP surface externally. The nanoparticles can be targeted to cancer cells through binding with nucleolin, a cancer cell membrane receptor. We will demonstrate representative results of human breast cancer cell targeting using these NPs.

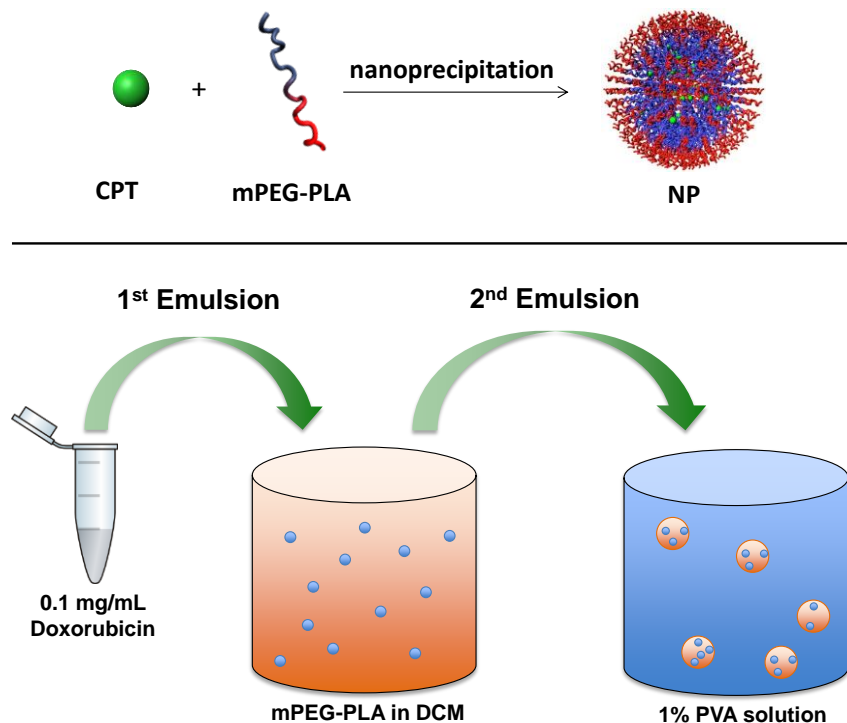


Figure 1. A schematic depiction of nanoprecipitation (top) followed by double emulsion (bottom).

Overview of cancer nanomedicine:

Polymeric nanoparticles for cancer therapy. In the first section of this module, nanoparticles (NPs) and microparticles (MPs) will be fabricated through two different synthetic routes. In the first experiment, an anticancer drug, camptothecin (CPT), will be encapsulated during the self-assembly of diblock polymer to form nanoparticles. mPEG-PLA, a diblock copolymer of methoxy-terminated polyethylene glycol (mPEG, hydrophilic) and polylactic acid (PLA, hydrophobic), will assemble in water to bury the hydrophobic PLA block while exposing PEG on the surface. This process, termed nanoprecipitation, is depicted in Figure 1a. CPT will be encapsulated in the interior of the nanoparticle.

In the second experiment of this section, a water-soluble anticancer drug, Doxorubicin, will be encapsulated through a double emulsion process (Figure 1b). In the first emulsion, a water-in-oil emulsion (W/O), an aqueous solution of Doxorubicin will be dispersed in the dichloromethane continuous phase. This will then be transferred to a polyvinyl alcohol (PVA) solution to create the second emulsion (W/O/W). PVA serves as an emulsifier to stabilize the mPEG-PLA microparticles. Subsequent dilution and stirring removes dichloromethane, resulting in the microparticles. In practice, the release rate of drug can be tuned by varying the amount of mPEG in the copolymer, which also affects the *in vivo* residence time in blood.¹

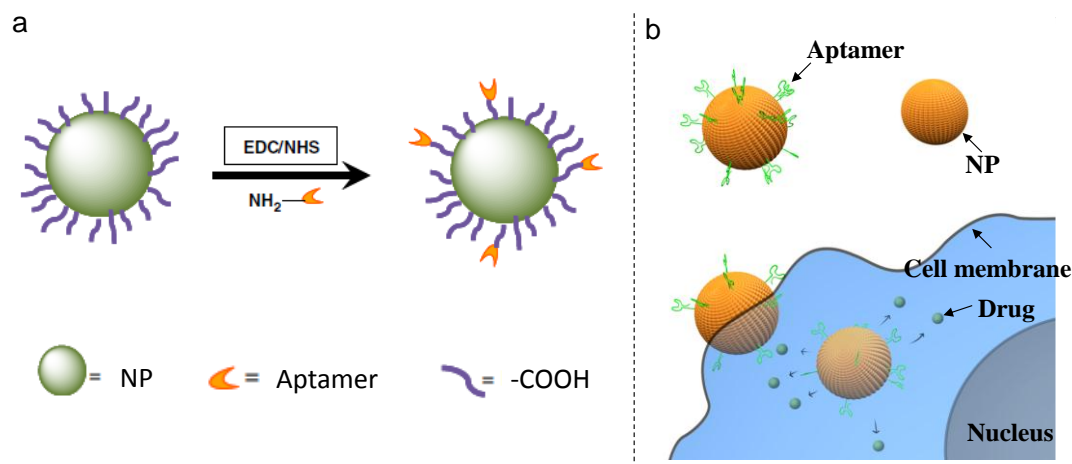


Figure 2. (a) Conjugation of aptamer to NP surface. (b) NP with aptamer modification can target cancer cells.

Aptamer for cancer targeting: Aptamers are oligonucleic acid or peptide molecules that bind to specific target molecules. We use an aptamer called AS1411, which can specifically target the nucleolin on the surface of breast cancer cells. This aptamer will be covalently conjugated to the NP surface using EDC/NHS chemistry² (section 2, Figure 2a). NP with surface modified by a random DNA sequence will be used as the control sample. These two NPs with fluorescence labeling will be incubated with 4T1 cells, a mouse breast cancer cell line (Figure 2b). To evaluate the targeting effect, the cells will be characterized by both a microplate reader (after cell lysis) and a fluorescence microscope.

Module outline and workflow:

The students are expected to gain an understanding of drug containing nanoparticles and their applications in cancer therapy. In section 1, students will first formulate both micron-size particles (MPs) and nanoparticles containing anticancer drugs. Relevant particle characterization techniques will be discussed. In section 2, they will then conjugate a targeting ligand, i.e. aptamer, to a fluorescence labeled nanoparticles and learn how to purify and sterilize the nanoparticles. In section 3, students will have the opportunities to apply these NPs to cancer cells and use fluorescence microscope to study the cancer cell targeting capability of these NPs.

Students will be divided to four groups (3-4 students each group) to do the experiments.

Section 1: MP and NP formulation.

Solution preparation

The following solutions will be used in the synthesis of NPs and MPs. Students will prepare them as part of this section unless otherwise noted.

- mPEG-PLA/DMF solution, 10mg/mL
- CPT/DMF solution, 0.5 mg/mL (stock solution prepared)
- mPEG-PLA/DCM solution, 50mg/mL
- 1% PVA solution (stock solution prepared)
- 0.1 mg/mL Doxorubicin solution (stock solution prepared)

Nanoparticle preparation by nanoprecipitation

A solution of the block copolymer mPEG-PLA will first be combined with the drug CPT. Then CPT will be encapsulated by the polymer during the self-assembly process in water. These particles will be used in subsequent sections of this module

1. CPT/DMF solution, 0.5 mg/mL (pre-prepared, solution **A**)
2. Dissolve 10 mg of mPEG-PLA in 1 mL DMF (solution **B**)
3. Mix 100 μ L solution **A** with 100 μ L solution **B** in microcentrifuge tube to become solution **C**
4. Prepare 4 mL DI water with stirring bar in a scintillation vial
5. Nanoprecipitation: Dropwise add solution **C** into stirring DI water solution

Microparticle preparation by double emulsion

PVA solutions will first be made in preparation to stabilize the W/O/W emulsions. Sonication will create the first emulsion (W/O) of aqueous Doxorubicin in the DCM solution of mPEG-PLA. The homogenizer will be used to create the second emulsion (W/O/W).

1. Pour out 15 mL of 1% PVA stock solution into a 50 mL beaker (solution **D**)
2. In a 100 mL beaker, mix an additional 15 mL of PVA stock solution with 35 mL DI water to create a 0.3% PVA solution (solution **E**)
3. In a microcentrifuge tube, dissolve 50 mg of mPEG-PLA in 1 mL of dichloromethane
4. Add 50 μ L of 0.1 mg/mL Doxorubicin solution
5. Sonicate the mixture for approximately 20 seconds
6. Quickly transfer the emulsion into solution **D**
7. Perform the second emulsion by mixing the solution with the homogenizer for 60 seconds
8. Pour the resulting emulsion into solution **E**
9. Stir the solution on a stir plate to allow dichloromethane to evaporate

Section 2: NP surface modification with aptamer.

Solution preparation

- Florescence NP solution (stock solution prepared)
- aptamer solution (stock solution prepared, solution **F**)
- random DNA solution (stock solution prepared, solution **G**)
- EDC/DI water solution, 10 mg/mL
- NHS/DI water solution, 10 mg/mL

Aptamer conjugation with nanoparticles

1. NP solution is diluted to 1 mg/mL by DI water (solution **H**)
2. Two scintillation vials are prepared with stirrer bars. For each of them: 200 μ L EDC solution and 200 μ L NHS solution are added to solution **H** (0.5 mL) to give solution **I**, which will be stirred for 10 min at RT.
3. For one of the vial, add 10 μ L of the aptamer solution (solution **F**) to solution **I**. The mixture will be further stirred for 1 hour to give NPs labeled by aptamer.
4. For the other vial, add 10 μ L of the random DNA solution (solution **G**) to solution **I**. The mixture will be further stirred for 1 hour. The mixture will be further stirred for 1 hour to give NPs modified by random DNA sequence as control.

NP purification and sterilization

1. NP solution will be purified by centrifuge (10,000 rpm, 5 min) and washing. (5mL X 3)
2. At last the NPs will be sterilized by using 0.4 μ m filter for use in cell culture.

Calibration curve

5. NP solution (solution **H**) will be sequentially diluted to different concentrations (1, 10, 50, 100, 500, 1000 μ g/ml)
3. The fluorescence intensity will be measured by plate reader for each concentration to get the calibration curve.

Section 3: Cancer cell targeting with NPs.

Preparation

1. NP modified with aptamer or random DNA will be pre-prepared (1mg/mL). Students will dilute these two solutions to 100 μ g/mL in sterile water in a biosafety cabinet. The NP solution will be further sterilized using a 0.4 μ m filter.
2. Each student will be provided with cells cultured in both 96 well plate (for lysis and bio-reader analysis) and chamber slide (for imaging and counting cells).
3. A sample of cell lysis will be pre-prepared by instructors to show the result of microplate reader (for step 5)
4. A sample of slides will be pre-prepared to show the students the microscope images of cells (for step 6)

Cell uptake experiment (all will be done in biosafety cabinet)

1. Before the experiment, basic principle of Module 3 is taught.
2. The students will learn how to treat cells with NPs. Here cells cultured in the 96 well plates will be provided to students. Students need to wash cells with PBS (1mL X 1), and then add opti-MEM (0.9mL/each well). They then add NPs to each well to bring the final concentration of NP to 10 μ g/mL and incubate the cells with NPs for four hours. (The last step, washing cells with PBS and adding lysis buffer are omitted due to time limitations.)
3. Due to the time limitations, students are provided with pre-prepared sample of cell lysis to learn how to use microplate reader for in vitro measurements of cellular uptake condition.
4. The students are then provided with pre-prepared chamber slide with cells already treated with NPs for four hours to learn to use a fluorescent microscope to observe the cellular uptake condition of different groups and take images.

Summary & conclusion:

In section 1, the students learned the formulation of drug conjugated polymeric nanoparticles, created through both nanoprecipitation and double emulsion strategies. In section 2, the students learned the surface modification method of conjugating aptamers onto nanoparticles tagged with FITC. In section 3, the students learned the effects of the aptamer conjugated nanoparticles on cancer cells. The students administered the ligand specific aptamer conjugated nanoparticles and random aptamer conjugated to the nanoparticles. The students observed the effects of the aptamers through qualitative analysis with fluorescence microscopy and quantitative analysis through the measure of fluorescence intensity with a microplate reader. Through this module, we are able to go through a whole process of preparing and testing nanoparticles for the purpose of testing its drug delivery and uptake capability in cells *in-vitro*.

References

1. Avgoustakis, K., *et al.* PLGA-mPEG nanoparticles of cisplatin: in vitro nanoparticle degradation, in vitro drug release and in Vivo drug residence in blood properties. *J Control Release* **79**, 123-135 (2002).
2. Cheng, J., *et al.* Formulation of functionalized PLGA-PEG nanoparticles for in vivo targeted drug delivery. *Biomaterials* **28**, 869-876 (2007).