Video Article

Synthesis of Gold Nanoparticle Integrated Photo-responsive Liposomes and Measurement of Their Microbubble Cavitation upon Pulse Laser Excitation

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URL: https://www.jove.com/video/53619
DOI: doi:10.3791/53619

Keywords: Bioengineering, Issue 108, Exogenous stimuli-responsive nanoparticles, light-responsive liposomes, gold nanoparticles, drug delivery, controlled release, microbubble cavitation

Date Published: 2/24/2016

Citation: Mathiyazhakan, M., Chan, W., Ohl, C.D., Xu, C. Synthesis of Gold Nanoparticle Integrated Photo-responsive Liposomes and Measurement of Their Microbubble Cavitation upon Pulse Laser Excitation. *J. Vis. Exp.* (108), e53619, doi:10.3791/53619 (2016).

Abstract

Photo-responsive nanoparticles (NPs) have received considerable attention because of their potential in providing spatial, temporal, and dosage control over the drug release. However, most of the relevant technologies are still in the development process and are unprocurable by clinics. Here, we describe a facile fabrication of these photo-responsive NPs with commercially available gold NPs and thermo-responsive liposomes. Calcein is used as a model drug to evaluate the encapsulation efficiency and the release kinetic profile upon heat/light stimulation. Finally, we show that this photo-triggered release is due to the membrane disruption caused by microbubble cavitation, which can be measured with hydrophone.

Video Link

The video component of this article can be found at https://www.jove.com/video/53619/

Introduction

The possibility to trigger drug release using external stimuli is an attractive way to deliver the drugs in spatial-, temporal- and dosage-controlled fashions with maximized specificity and minimal adverse effects. Among a wide range of exogenous stimuli-responsive systems (light, magnetic field, ultrasound, microwave radiation), light-triggered platforms are attractive, owing to their non-invasiveness, simplicity and adaptability in the clinics.[1] Extensive research in the past decade has provided a variety of platform technologies, such as near-infrared-light responsible gold (Au) nanocages coated with smart polymers,[2] photo-labile, polymeric nanoparticles (NPs) conjugated with drugs,[3] and self-assembled porphysome nanovesicles.[4] However, these technologies are still in the preclinical stages of development, and require a clear understanding and optimization of parameters involved in the process of initiating and controlling the drug release.

One of the simplest and easily accessible methods for preparing such a system is to integrate Au NPs with thermally-sensitive liposomes[5,6], both of which are widely available in the market and have been extensively investigated in preclinical and even clinical trials. Despite the limitation of deep-tissue activation of Au NPs at their plasmonic wavelength, when compared to near-infrared-activated Au nanostructures (*e.g.,* nanocages), this system still holds great promise when used in small animals or for topical delivery in humans.[7] There are some early efforts in combining Au NPs with liposomes for light-triggered release.[8-11] While most of them focus on the novelty of materials, accessibility and scalability issues need to be addressed. Moreover, reports on release mechanisms using these nanocarriers are still limited.

Herein, the fabrication of photo-responsive liposomes, simultaneously loaded with drugs and hydrophilic Au NPs has been described. Calcein is used as a model compound to evaluate the encapsulation efficiency and the release profile of the system. In addition, in this system, light absorbed by Au NPs dissipates to the surrounding microenvironment in the form of heat, resulting in an increase in the local temperature. Air microbubbles are generated during the laser heating and cause mechanical disruption of liposomes (**Figure 1**). The mechanism of microbubble cavitation is confirmed by hydrophone measurements.

Protocol

1. Preparation

1. Clean 100 ml round bottom flasks using aqua regia (1 part of concentrated nitric acid (HNO_3) and 3 parts of concentrated hydrochloric acid (HCl)) and wash the flasks with DI water. Autoclave the flasks and dry them in a hot air oven at 100 °C for 15 min. Wrap and store the sterile flasks until use.

2. Sterilize the hand-held mini-extruder set using 70% ethanol.
3. Turn on the rotary evaporator and set the temperature of the hot water bath and the cooling tower at 37 °C and 4 °C respectively.
4. Prepare 60 mM calcein stock solution by dissolving 374 mg of calcein in 10 ml of 0.1 mM phosphate buffered saline (PBS) (pH 7.4). Adjust the pH to 7.4 using 1 M sodium hydroxide (NaOH) solution.

2. Synthesis of Liposomes

1. Remove the lipids (1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-hydroxy-*sn*-glycero-3-phosphocholine (MPPC) and 1, 2-distearoyl-*sn*-glycero-3-phosphoethanol-amine-*N*-[carboxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG2000)) from freezer (-20 °C) and thaw them to RT.
2. Weigh 15.9 mg DPPC, 1.3 mg MPPC, and 2.8 mg DSPE-PEG2000. Dissolve them together in 2 ml chloroform.
3. Transfer the chloroform solution to the sterile round bottom flask and evaporate the solvent using the rotary evaporator under reduced pressure, to form a thin, dry lipid layer.
4. Hydrate the lipid layer at 45 °C with 2 ml aqueous solution for 30 min containing 1.95 ml 60 mM calcein prepared in step 1.4 and 50 µl Au NPs (3.36×10^{16} particles/ml).
5. Pre-heat the heating block to 45 °C. Place a 200 nm polycarbonate membrane filter between the filter supports and assemble the mini-extruder set. Check the potential leakage with DI water.
6. Fill one of the syringes with 1 ml of liposome solution from step 2.4 and extrude the sample by passing the solution to the syringe at the other end of the assemble mini-extruder. Repeat 11 times.
7. Run the synthesized liposomes through a PD-10 desalting column to remove the free Au NPs, lipids and calcein using PBS as the eluent according to manufacturer's protocol.
8. Store the samples in sterile tubes at 4 °C and use in 2 days.

3. Calcein Release from Liposomes with Heating

1. Calculate the lipid molarity of the stock solution by adding up the molarities of DPPC, MPPC, and DSPE-PEG2000 in step 2.2. Dilute the liposome stock solution to 5 mM lipid concentration using 0.1 mM PBS buffer (pH 7.4). Transfer the samples to a centrifuge tube (2 ml).
2. Place the tube in a hot water bath, and raise the temperature gradually from 25 to 70 °C. Increase the temperature at a rate of 1 °C/min here.
3. Collect aliquots (10 µl) at different temperature points (27, 32, 37, 39, 41, 43, 45, 52, 57, 62, 67 and 70 °C).
4. Add 10 µl of 2% Triton X-100 to 2 ml aliquot of liposomal solution to digest the liposomes for 10 min at RT and to achieve complete release of calcein.
5. Transfer 200 µl of the liposomal solution to each well in the 96-well microplate and measure the fluorescence intensity of the collected samples using a fluorescence microplate reader. The excitation and emission wavelengths of calcein are 480 and 515 nm respectively.
6. Taking the fluorescence intensity of the Triton X-100 treated samples as 100% release, calculate the percentage of the released calcein at each time point, using the formula:

$$\%\ Release = 100 \times \frac{F_t - F_i}{F_x - F_i}$$

Ft is the fluorescence intensity of solution at a given time point. F_i and F_x are the normalized initial and final fluorescence intensities of the solution respectively.

4. Calcein Release from Liposomes with Pulsed Laser

1. Transfer 100 µl liposome solution to a quartz cuvette and place it in a cuvette holder. Use a pulsed Nd:YAG laser with a pulse duration of 6 nsec at 532 nm wavelength as the light source. Use the following Laser Parameters — Repetition rate: 1 Hz; Laser energy density: 1 mJ/cm^2; Beam diameter: 0.5 mm.
2. Guide the collimated laser beam through the cuvette such that the light passes through the liposome solution and collect aliquots after various pulses.
3. Add 10 µl of 2% Triton X-100 to 2 ml aliquot of liposomal solution to digest the liposomes for 10 min at RT and to achieve complete release of calcein.
4. Considering calcein is sensitive to light and could be bleached during the laser experiment, pre-measure the bleaching effect of pulsed laser on calcein solution to normalize the data from liposome release.
    1. Specifically, expose calcein solution (60 mM) to pulsed laser with frequency of 1 Hz for varying pulse numbers (0, 25, 50 and 100). Measure the fluorescence intensity of calcein with excitation and emission wavelengths of 480 and 515 nm respectively, before and after laser exposure.
    2. Calculate the amount of bleaching (fluorescence intensity of calcein before laser exposure/ fluorescence intensity of calcein after the specific pulse number). Use this factor to normalize the values obtained from liposome samples by multiplying the fluorescence intensity of the liposomes with the factor.
5. Measure the fluorescence intensity of the collected sample using a fluorescence microplate reader at excitation and emission wavelengths at 480 and 515 nm respectively.
6. Taking the fluorescence intensity of the Triton X-100 treated samples as 100% release, calculate the percentage of the released calcein at each pulse number, using the formula:

$$\% \, Release = 100 \times \frac{F_t - F_i}{F_x - F_i}$$

Ft is the fluorescence intensity of solution at a given pulse number after normalization. F_i and F_x are the normalized initial and final fluorescence intensities of the solution respectively.

5. Measurement of Pressure Impulses

1. Place 100 µl of the sample on a microscopic slide and set the focus of the laser onto the sample.
2. Immerse a needle hydrophone (1 mm diameter and 450 nV/Pa sensitivity) into the solution.
   Caution: The hydrophone must not be illuminated by the laser light to avoid the damage.
3. Irradiate the sample with the pulsed laser with varying pulse numbers (0-100) and pulse energy (20-160 µJ/pulse).
4. Record the pressure signals using a digital oscilloscope.

Representative Results

Liposomes were prepared using a conventional thin film hydration technique with DPPC, MPPC and DSPE-PEG2000 in a molar ratio of 86:10:4 or 7.95:0.65:1.39 mg/ml.[12] The size of Au NPs is critical to determine the light to heat conversion efficiency during the following laser excitation experiment. Smaller the size of Au NPs, higher is the transducing efficiency.[13] Thus 5 nm Au NPs, the smallest samples from the vendor, were chosen for encapsulation. During the synthesis, hydrating medium containing Au NPs and calcein was added to generate multi-lamellar vesicles, which were then subject to size extrusion and gel filtration chromatography to remove the free Au NPs and calcein.

Calcein is a hydrophilic fluorescent dye is encapsulated within the aqueous core of the liposomes at a concentration of 60 mM. At such high concentrations, the fluorescence of calcein self-quenches. However, calcein is diluted as it is released from the liposomes, leading to fluorescence re-gain, indicating triggered drug release.[14] Relying on this property of calcein liposomes were diluted to 5 mM and the response of the liposomal system to the temperature change was observed. As indicated in **Figure 2A,** regardless of the presence of Au NPs, the liposomes were almost intact at the physiological temperature (*i.e.,* 37 °C) with a leakage percentage of less than 10%. However, when the temperature was raised to 42 °C (slightly above the transition temperature of lipids), 60%-80% of the encapsulated calcein is released from liposomes within 2 min. No significant difference in the release percentage is observed within the liposomes with and without Au NPs, when the release is triggered using heat. This is due to the phase transition temperature of the system. Moreover, the quick and efficient release is due to the presence of 10% MPPC, which enhances the permeability of liposome bilayer at the transition temperature.

Next, the calcein release from liposomes upon laser irradiation was examined. The liposomal solution was taken in a quartz cuvette which was placed in a cuvette holder. 532 nm Nd:YAG pulse laser with a pulse duration of 6 nsec and an instantaneous power density of 166.67 kW/cm^2 was focused onto the liposomal solution. Aliquots of samples were collected at varying pulse numbers (0, 25, 50, and 100) in which the pulse frequency was fixed at 1 Hz. The samples were immediately transferred to a microcentrifuge tube and placed in an ice bath. This is to prevent any further release of calcein. The laser was turned off while collecting the samples. As shown in **Figure 2B**, liposomes with Au NPs released the encapsulated calcein upon excitation, in which the amount of released calcein increased as the pulse numbers increased.

The last step was to study the mechanism of calcein release from the liposomes with hydrophone. **Figure 3A** is the acoustic signal recorded for Au NPs (red), liposomes with Au NPs (black) and liposomes without Au NPs (blue). While Au NPs and liposomes with Au NPs showed characteristic pressure wave signals, no signal was observed from liposomes without Au NPs. The pressure impulse contains both positive and negative phases (inset in **Figure 3A**). It suggests that microbubbles form (positive phase) and disrupt (negative phase) in the solution. The maximum and the minimum values of the recorded peak in the Au NP-containing liposomes were 0.00093 and -0.00074 V respectively. Finally, the average maxima and minima of the pressure impulses as a function of increasing laser energy were calculated. As expected, the acoustics signal amplitude gradually increased with the increasing laser energy (**Figure 3B**). This should be due to the increased intensity of the oscillation of the particles attributing to the subsequent thermo-elastic expansion and contraction.[15,16]

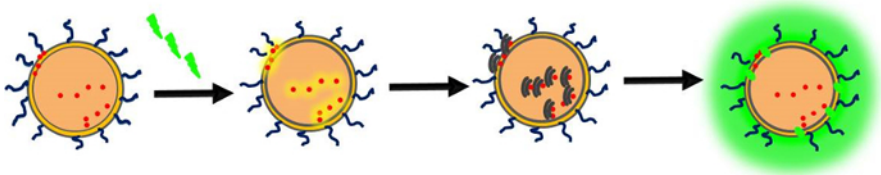

Figure 1. The proposed drug release mechanism of photo-responsive liposomes: Au NPs (red dots) when being irradiated, generate microbubbles that disrupt the liposome membrane and thus trigger calcein release. Calcein, self-quenching within liposomes (as shown), fluoresces as being released to the surrounding. Please click here to view a larger version of this figure.

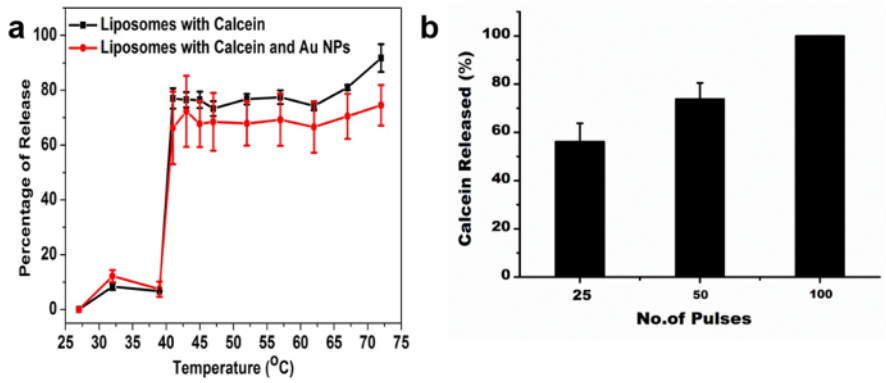

Figure 2. Heat and pulsed laser triggered release of calcein from liposomes. (**A**) Percentage of released calcein from liposomes with or without Au NPs, which were placed in water bath with various temperatures for 2 min; (**B**) Percentage of released calcein from liposomes with Au NPs, which were treated with pulsed laser (pulse duration = 6 nsec; power density ~12 mW/cm^2). Error bars represent the mean ± standard deviation (SD) of three independent experiments performed in triplicate. Please click here to view a larger version of this figure.

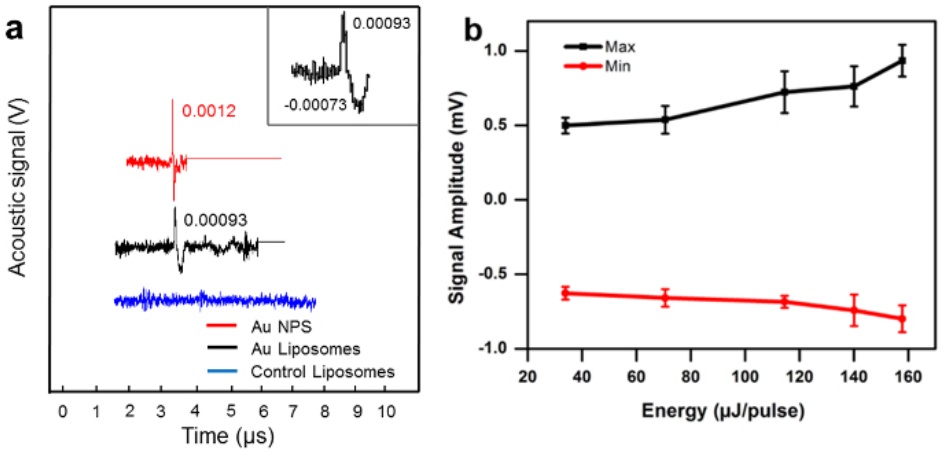

Figure 3. (**A**) Acoustic signals measured using a hydrophone system for Au NPs (red), liposomes with Au NPs (black) and liposomes without Au NPs (blue). While Au NPs and liposomes with Au NPs showed characteristic pressure wave signals, no signal was observed from liposomes without Au NPs. The inset shows an enlarged view of acoustic signals of photo-responsive liposomes. (**B**) Maximum and minimum peak value of the pressure impulses as a function of increasing laser power. The acoustics signal amplitude gradually increases with the increasing laser energy. Error bars represent the mean ± SD of three independent experiments performed in ten runs. Please click here to view a larger version of this figure.

Discussion

Thin film hydration is the conventional method for preparing liposomes. Organic solvents (chloroform in this case) were first used to dissolve the lipids and then removed in a rotary evaporator at 37 °C to generate a lipid thin film on the flask. This lipid film was hydrated with the aqueous solution containing 60 mM calcein and 5 nm Au NPs. During the hydration process, the temperature was maintained around 50 °C and the flask was constantly agitated by rotating the flask. The key in this step is the choice of temperatures at which the evaporation and hydration were carried out respectively. The phase transition temperature (T_m) of DPPC, the major constituent of the liposome, is 41 °C. During the formation of the lipid think film, the temperature should be below 41 °C to prevent the formation of aggregated clumps of dried lipids. During the hydration, a higher temperature than T_m was chosen to drive the phospholipids to self-assemble into spherical multilamellar structures. Although thin film hydration method is easy to perform, it is limited by poor encapsulation efficiency (<1%) of the calcein and Au NPs in the aqueous solution. In the future, the encapsulation might be improved through freeze-thaw cycles.

Subsequent size-extrusion was done using a hand held mini extruder, placed on a heating block. A maximum of 1 ml solution can be extruded using this set-up, which is ideal for a small scale preparation in the laboratory. The key in this step is still the temperature, which should be above the phase transition temperature of liposomes. By handing the extrusion at 50 °C, the size of liposomes became uniform after 10 cycles of extrusion through the membrane with 200 nm pores. The motion in this step should be slow and gentle as the pressure within the extruder is big while the membrane is fragile.

One often-overlooked but important step during the synthesis of drug containing liposomes is the purification or removal of the un-encapsulated drugs (*i.e.,* calcein and Au NPs here). This was done by gel filtration chromatography. It is critical for the researcher to notice that the PD-10 column used in this study is only suitable for processing <2 ml solution. Samples with larger volume could be processed by using multiple columns.

The study of calcein release from the liposome relies on the self-quenching of calcein at higher concentrations and re-fluorescence as diluted. When the samples were exposed to heat or light for thermal or laser treatment respectively, aliquots of samples were constantly withdrawn at varying temperature points or pulse numbers and transferred to separate vials. The vials should be immediately placed in an ice bath to prevent the potential further release of calcein, for accurate measurements. Another thing to notice is that calcein is sensitive to light and hence could be bleached during the laser experiment. To overcome this, the bleaching effect of a pulsed laser on the calcein solution should be measured and the data from liposome release normalized accordingly.

The protocol herein describes a facile preparation method for light sensitive liposomes. Thermal release is first demonstrated by exploiting the temperature responsive of the phospholipids. The laser set up is illustrated and light triggered release is achieved by pulsed laser. The mechanism of the light triggered release is also explored and is found to be due to membrane disruption by microbubble cavitation.

Different from all reported protocols, this protocol chooses materials (*i.e.,* lipids and Au NPs) that have been frequently used in clinical trials and widely available in the market. The use of readily accessible materials allows anyone to prepare such light-sensitive system by themselves, without the need to seek technical help. In addition, this protocol makes use of thin film hydration to prepare liposomes, which is both cost-effective and easily scalable.

This simple but powerful protocol will benefit researchers and clinicians who are interested in achieving controlled drug delivery to superficial tissues like skin. One potential application would be to deliver anti-sunburn agents by incorporating this system within sunscreen.

Disclosures

No conflict of interest are declared.

Acknowledgements

This work was partially supported by the Tier-1 Academic Research Funds by Singapore Ministry of Education (RG 64/12 to CX) and NTU-Northwestern Institute of Nanomedicine.

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