

Video Article

Synthesis and Characterization of Placental Chondroitin Sulfate A (pICSA)-Targeting Lipid-Polymer Nanoparticles

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Abstract

An effective cancer therapeutic method reduces and eliminates tumors with minimal systemic toxicity. Actively targeting nanoparticles offer a promising approach to cancer therapy. The glycosaminoglycan placental chondroitin sulfate A (pICSA) is expressed on a wide range of cancer cells and placental trophoblasts, and malarial protein VAR2CSA can specifically bind to pICSA. A reported placental chondroitin sulfate A binding peptide (pICSA-BP), derived from malarial protein VAR2CSA, can also specifically bind to pICSA on cancer cells and placental trophoblasts. Hence, pICSA-BP-conjugated nanoparticles could be used as a tool for targeted drug delivery to human cancers and placental trophoblasts. In this protocol, we describe a method to synthesize pICSA-BP-conjugated lipid-polymer nanoparticles loaded with doxorubicin (pICSA-DNPs); the method consists of a single sonication step and bioconjugate techniques. In addition, several methods for characterizing pICSA-DNPs, including determining their physicochemical properties and cellular uptake by placental choriocarcinoma (JEG3) cells, are described.

Video Link

The video component of this article can be found at <https://www.jove.com/video/58209/>

Introduction

An effective cancer therapeutic method reduces and eliminates tumors with minimal systemic toxicity. Hence, selective tumor targeting is the key to exploring successful therapeutic methods. Nanoparticles offer a promising opportunity for cancer therapy, and molecular assemblies with different functional groups will enhance drug efficacy and reduce associated side effects^{1,2}. Moreover, nanoparticle systems mainly utilize passive and active targeting to reach target tumors³.

Passive targeting exploits the innate characteristics of nanoparticles and enhanced permeability and retention (EPR) effects to reach tumor cells. Cationic liposomes have been successfully used to deliver various anticancer drugs to tumors in clinical applications^{4,5,6}. Despite the potential effective cancer therapeutic effect, a low drug concentration in the tumor region and an inability to distinguish tumor cells from normal tissues are two major limitations of passive-targeting nanoparticles⁷.

Active targeting strategies take advantage of antigen-antibody, ligand-receptor and other molecular recognition interactions to specifically deliver drugs to tumors⁸. The glycosaminoglycan placental chondroitin sulfate A (pICSA) is broadly expressed on most cancer cells and placental trophoblasts. Moreover, the malarial protein VAR2CSA can specifically bind to pICSA^{9,10}. Hence, VAR2CSA can be a tool for targeting human cancer cells. However, when VAR2CSA is conjugated to nanoparticles, the full-length protein may limit the penetration of nanoparticles into tumor cells. Recently, we discovered a pICSA binding peptide (pICSA-BP), derived from the malarial protein VAR2CSA. pICSA-BP-conjugated lipid-polymer nanoparticles rapidly bonded to choriocarcinoma cells and significantly increased doxorubicin (DOX) anticancer activity *in vivo*¹¹; these particles also specifically bonded to placental trophoblasts and could serve as a tool for the targeted delivery of drugs to the placenta¹².

Lipid-polymer nanoparticles consist of a lipid monolayer shell and a hydrophobic polymeric core and represent a new carrier for drug delivery. These nanoparticles combine the advantages of liposomes and polymeric nanocarriers, such as controllable nanoparticle size, high biocompatibility, sustained drug release, high drug loading efficiency (LE), and excellent stability¹³. In this work, we used a single-step sonication method to synthesize lipid-polymer nanoparticles. This method is fast, convenient and suitable for scale-up and has been widely used to prepare lipid-polymer nanoparticles by our group^{11,14} and others^{15,16,17,18}.

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) is a popular carbodiimide used as a crosslinking agent for conjugating biomolecules containing amines and carboxylates¹⁹. In addition to EDC, N-hydroxysulfosuccinimide (NHS) is the most common conjugation

reagent in surface and nanoparticle conjugation reactions^{20,21}. NHS can reduce the number of side reactions and enhance the stability and yield of ester intermediates^{22,23}.

Here, we describe a protocol for synthesizing pICSA-targeted lipid-polymer nanoparticles. First, the single-step sonication synthesis of DOX-loaded lipid-polymer nanoparticles (DNPs) is described. Then, an EDC/NHS bioconjugate technique for generating pICSA-BP-conjugated lipid-polymer nanoparticles is introduced. This bioconjugate technique can also be used to conjugate other antibodies and peptides to nanoparticles. Finally, we describe the physicochemical properties and *in vitro* assay used to characterize the pICSA-targeted lipid-polymer nanoparticles. We believe that these pICSA-targeted lipid-polymer nanoparticles could constitute an effective system for the targeted delivery of drugs to most human cancers and the targeted delivery of payloads to the placenta to treat placental disorders.

Protocol

1. Preparation of Stock Solutions

1. Prepare an aqueous solution of 4% ethanol by diluting 4 mL of absolute ethanol with 100 mL of ultrapure water. Store the solution at 4 °C. NOTE: Ultrapure water is defined as water without contaminants such as bacteria, particulates, ions, or nucleases. Ultrapure water was obtained from a water purification system with a target resistivity of up to 18.2 mΩ·cm, which means low anionic contamination.
2. Prepare a 1 mg/mL soybean lecithin stock solution by dissolving 20 mg of soybean lecithin in 20 mL of the aqueous solution of 4% ethanol. Store the soybean lecithin stock solution at 4 °C.
3. Prepare a 25 mg/mL DSPE-PEG (2000)-COOH stock solution by dissolving 100 mg of DSPE-PEG-COOH in 4 mL of the aqueous solution of 4% ethanol. Store the stock solution at -20 °C.
4. Prepare a 10 mg/mL DOX stock solution by dissolving 50 mg of DOX in 5 mL of ultrapure water. Store the DOX stock solution at 4 °C in the dark.
5. Prepare a 2 mg/mL PLGA stock solution by dissolving 20 mg of PLGA in 10 mL of acetonitrile. Store the PLGA stock solution at 4 °C. CAUTION: Acetonitrile is flammable and toxic. Operate with care in a fume hood, and wear appropriate personal equipment, such as lab coats, safety glasses and latex gloves.
6. Prepare a 0.1 M 2-(morpholino) ethanesulfonic acid (MES, pH 6.0) stock solution by dissolving 2.17 g of MES in 100 mL of ultrapure water. Store the stock solution at 4 °C.

2. Synthesis of DNPs

NOTE: To avoid DOX photochemical degradation, all operations were performed in the dark. Nanoparticles were synthesized by a previously reported single-step sonication method^{11,13,14}.

1. Add 3 mL of the aqueous solution of 4% ethanol to a sterile 10 mL centrifuge tube. Then, add 90 µg of the soybean lecithin stock solution, 210 µg of the DSPE-PEG-COOH stock solution and 750 µg of the DOX stock solution to 3 mL of the aqueous solution of 4% ethanol.
2. Place the centrifuge tube into an ice bath, and place the ice bath on an ultrasonic processor.
3. With a 1 mL syringe, pipette 2 mg of the PLGA stock solution dropwise (1 drop/4-6 s) into the centrifuge tube. Meanwhile, sonicate the tube using an ultrasonic processor at a frequency of 20 kHz and an output amplitude of 30% for 5 min to synthesize the DNPs. NOTE: To synthesize uniform particles with small sizes, the speed at which the PLGA solution is dripped into the tube needs to be slow, and bubble generation should be avoided.
4. Purify the DNPs by washing the above solution in 0.1 M MES buffer (pH 6.0) 3 times using a centrifuge filter (MWCO, 10 kDa). Centrifuge at 4 °C and 1000 × g for 3 min each time. Finally, approximately 1 mL of MES-solved nanoparticles should remain. NOTE: This is an acceptable stopping point in the procedure. If not used to conjugate peptides, the nanoparticles can be purified by PBS buffer (pH 7.4), and the purified DNPs can be stored at 4 °C in the dark.

3. Conjugation of Peptides to DNPs

1. **Ester activation**
 1. Add 0.4 mg of EDC (final concentration 2 mM) to 1 mL of DNPs.
 2. Add 0.24 mg of NHS to the reaction (final concentration 2 mM). NOTE: For easy addition of the correct quantity of EDC and NHS, a stock solution may be prepared if the reagents are dissolved and used immediately.
 3. Mix the reaction components well, and place the reaction on a shaker; allow reaction for 30 min-1 h at room temperature in the dark.
2. **Amine reaction**
 1. Increase the buffer pH to 7.2-7.5 using PBS (20×, pH 7.4).
 2. Add 0.5 mg of the pICSA-targeting peptide (pICSA-BP, EDVKDINFDTKEKFLAGCLIVSFHEGKC) to the reaction solution. NOTE: Before addition, dissolve the peptides in 20% acetonitrile. If peptides are not dissolvable, sonication in a bath sonicator may be helpful.
 3. Mix the solution well, and then place on a shaker; allow the reaction to proceed at 4 °C overnight in the dark.
 4. Place conjugate solution into dialysis bags (MWCO, 3,500 Da) to dialyze and purify the pICSA-DNPs using PBS (pH 7.4) buffer at room temperature for 24 h in the dark. NOTE: Alternatively, purification could be performed as in step 2.4 to obtain pICSA-DNPs.
 5. For cell culture applications, filter the reconstituted solution through a 0.22 µm sterile syringe filter to remove potential precipitates.

4. Characterization of pICSA-Targeted Lipid-Polymer Nanoparticles

1. Measurement of the hydrodynamic nanoparticle size using dynamic light scattering (DLS)

1. Dilute nanoparticles with ultrapure water (50-fold dilution). Load 500 μL of the sample into a cuvette according to the instructions of the DLS or zeta potential instrument.
NOTE: Zeta potential and DLS cuvettes can differ based on the instrument's specifications.
2. After the measurement is completed, record the particle diameter, polydispersity index (PDI) and zeta potential. Average the results obtained from 4 repeated readings, and calculate the standard deviation.

2. Transmission electron microscopy (TEM)

NOTE: The morphology of the nanoparticles was observed by TEM with the negative stain method.²⁴

1. Dilute nanoparticles with ultrapure water (400-fold dilution). Add 20 μL of the sample onto a TEM grid, and allow to sit for 5 min.
2. Add 100 μL of 2% (w/v) phosphotungstic acid and allow to sit for 2 min. Wick away the droplet.
3. Dry the TEM grid at room temperature.
4. Set the TEM acceleration voltage at 80 kV, and magnify the image to 100,000 \times to visualize the nanoparticles.

3. Determination of the encapsulation efficiency (EE) and LE

1. Standard curve generation. Dissolve DOX in ultrapure water to prepare DOX solutions of five different concentrations: 1 $\mu\text{g/mL}$, 5 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$, 50 $\mu\text{g/mL}$, and 100 $\mu\text{g/mL}$. Measure the absorption of the DOX solutions at 480 nm with a UV-VIS spectrometer. Generate a standard curve based on the DOX concentrations.
2. Dilute 25 μL of nanoparticles with 500 μL of ultrapure water. Measure the absorption at 480 nm with a UV-VIS spectrometer. Calculate the drug concentrations by the standard curve.
3. Calculate the LE using the following equation:
$$\text{LE} = ((\text{amount of drugs in nanoparticles}) / (\text{total weight of materials})) \times 100\%$$
4. Calculate the EE using the following equation:
$$\text{EE} = ((\text{amount of drugs in nanoparticles}) / (\text{amount of added drug})) \times 100\%$$

4. Measurement of the conjugation efficiency using the bicinchoninic acid (BCA) assay^{25,26,27}

1. Pipette 25 μL of standard peptide solutions (Table 1) or pICSA-DNPs into microplate wells in duplicate. Add 200 μL of the working reagent to each well, and mix the plate well on a plate shaker for 30 s.
2. Cover the plate, and incubate at 37 $^{\circ}\text{C}$ for 30 min.
3. Measure the absorbance at 562 nm on a plate reader. Use the generated standard curve to calculate the pICSA concentrations of pICSA-DNPs.
4. Calculate the conjugation efficiency using the following equation:
$$\text{Conjugation efficiency} = ((\text{amount of peptide in nanoparticles}) / (\text{amount of added peptide})) \times 100\%$$

| Vial | Volume of diluent (μL) | Volume and source of peptide (μL) | Final peptide concentration ($\mu\text{g/mL}$) |
|------|-------------------------------------|--|--|
| A | 0 | 300 of Stock | 1000 |
| B | 250 | 250 of vial A dilution | 500 |
| C | 250 | 250 of vial B dilution | 250 |
| D | 250 | 250 of vial C dilution | 125 |
| E | 300 | 200 of vial D dilution | 50 |
| F | 250 | 250 of vial E dilution | 25 |
| G | 400 | 100 of vial F dilution | 5 |
| H | 500 | 0 | 0 |

Table 1. Preparation of Standard peptides

5. Fluorescence Microscopy Assessment of pICSA-Targeted Nanoparticle Uptake in Choriocarcinoma (JEG3) Cells

1. Seed cells onto sterile 12-well plates at 1.0×10^4 cells/well with complete DMEM/F12 (cDMEM/F12, containing 1% penicillin/streptomycin and 10% fetal bovine serum (FBS)). Allow the cells to grow to 60% confluence at 37 $^{\circ}\text{C}$ and 5% CO_2 under humid condition.
2. Remove the media, and add 1 mL of cold fresh media with a low serum content (5% FBS) and DNPs or pICSA-DNPs (5 μg of DOX equivalent).
3. Incubate the mixture of cells and nanoparticles at 4 $^{\circ}\text{C}$ for 1 h.
4. After incubation, remove the media, and wash the cells three times with PBS. Then, add 1 mL of fresh cDMEM/F12, and incubate the cells at 37 $^{\circ}\text{C}$ for 30 min.
5. Remove the cDMEM/F12, and wash the cells three times with PBS.
6. Add 2 mL of cold 4% paraformaldehyde (PFA), and incubate at room temperature for 15 min to fix the cells.
7. Remove the PFA. Wash the cells with 2 mL of PBS once. Add 1 mL of PBS containing DAPI (1 $\mu\text{g/mL}$) for nuclei staining, and incubate at room temperature for 10 min.

8. Aspirate the PBS, and wash the cells three times with PBS.
9. Add mounting medium, and image the fluorescence with a fluorescence microscopy, using green and blue channels to visualize the DOX and nuclei, respectively.

Representative Results

In this protocol, PLGA, DSPE-PEG-COOH and soybean lecithin are a representative polymer, lipid-PEG-COOH conjugate and lipid, respectively. The synthesis of pICSA-targeted lipid-polymer nanoparticles *via* a single-step sonication method and an EDC/NHS technique is illustrated in **Figure 1**. First, under sonication conditions, soybean lecithin, PLGA and DSPE-PEG-COOH self-assemble to form core-shell structured DNPs. The core consists of PLGA and encapsulated DOX, the shell consists of DSPE-PEG-COOH, and the soybean lecithin monolayer is located at the interface of the shell and the core. Then, the -COOH groups exposed on the DNP surface were conjugated with the -NH₂ groups of the peptide *via* the EDC/NHS technique to synthesize pICSA-DNPs.

The DNPs prepared in this protocol were 82.3 ± 4.7 nm in diameter, and after conjugation to pICSA-BP, the diameter of the primary DNPs increased to 109.3 ± 5.9 nm (**Figure 2**). The PDI of the DNPs and pICSA-DNPs was 0.127 ± 0.005 and 0.134 ± 0.065 , respectively. TEM images of the DNPs and pICSA-DNPs also showed that particles were well dispersed and generally had spherical morphologies (**Figure 3**). The zeta potential of the DNPs and pICSA-DNPs was -20.1 ± 1.32 mV and -29.9 ± 3.56 mV, respectively (**Table 2**). Hence, these nanoparticles are predicted to be highly stable.

| Nanoparticles | Diameter(nm) | PDI | Zeta potential (mV) |
|---------------|-----------------|-------------------|---------------------|
| DNPs | 82.3 ± 4.7 | 0.127 ± 0.005 | -20.1 ± 1.32 |
| pICSA-DNPs | 109.3 ± 5.9 | 0.134 ± 0.065 | -29.9 ± 3.56 |

PDI#polydispersity index. Data are presented mean \pm SD (n = 3).

Table 2. Characterization of nanoparticles

The EE and LE of nanoparticles are important for clinical applications. The EE of DOX by the DNPs and pICSA-DNPs was $40.3 \pm 1.67\%$ and $39.5 \pm 1.94\%$, respectively. The LE of DOX in the DNPs and pICSA-DNPs was $6.2 \pm 0.74\%$ and $5.1 \pm 0.42\%$, respectively. These data showed that the extent of drug loading and drug encapsulation was maintained after pICSA-BP decoration. The decoration efficiency of pICSA-BP on the surface of DNPs was determined by the BCA assay (**Figure 4**). The pICSA-BP conjugation efficiency was found to be $45.5 \pm 3.7\%$.

The JEG3 cellular uptake assay indicated that the pICSA-DNPs rapidly bound to JEG3 cells within 30 min (**Figure 5**). Therefore, pICSA-BP was efficiently conjugated to the DNP surface in this protocol. Moreover, pICSA-BP could rapidly bind to JEG3 cells and increase the cellular uptake of the nanoparticles.

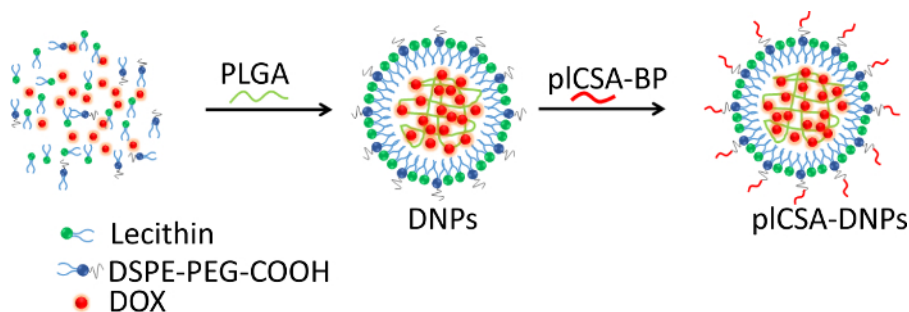


Figure 1. Schematic illustration of the method used to synthesize pICSA-targeted lipid-polymer nanoparticles. First, a single-step sonication method was used to synthesize lipid-polymer nanoparticles (DNPs), and then the EDC/NHS technique was used to conjugate peptides to the nanoparticle surfaces (pICSA-DNPs). [Please click here to view a larger version of this figure.](#)

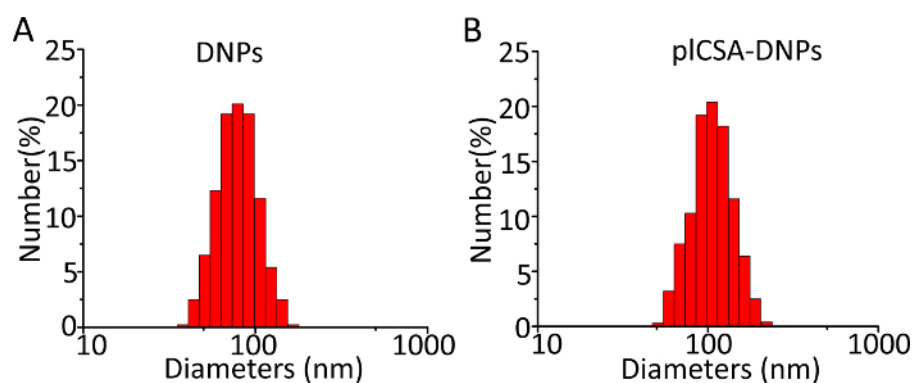


Figure 2. Size distribution of the different nanoparticles. Hydrodynamic size of DNPs (A) and pICSA-DNPs (B) measured by DLS. Representative figures are presented to demonstrate the particle size and size distribution. [Please click here to view a larger version of this figure.](#)

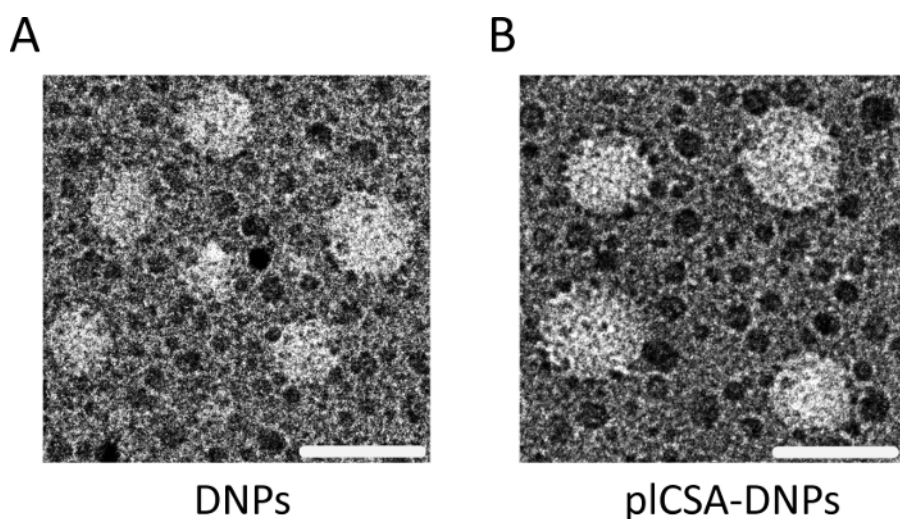


Figure 3. Nanoparticle morphology characterized by TEM. Typical TEM image of DNPs (A) and pICSA-DNPs (B) observed with 2% phosphotungstic acid staining. Scale bar=100 nm. [Please click here to view a larger version of this figure.](#)

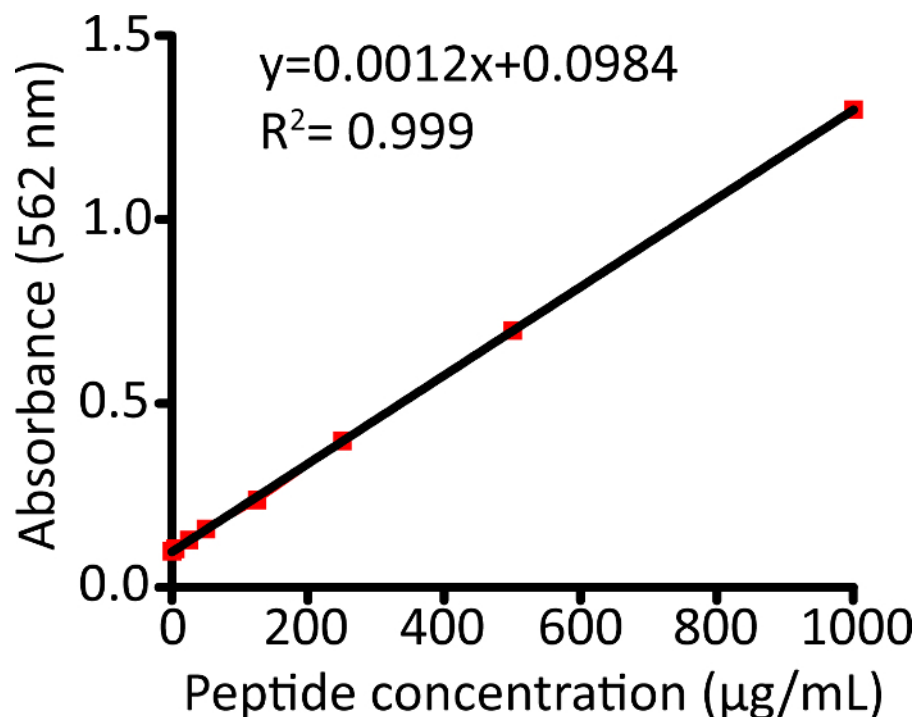


Figure 4. Measurement of the conjugation efficiency using the BCA assay. Standard curve of the peptides at 562 nm. [Please click here to view a larger version of this figure.](#)

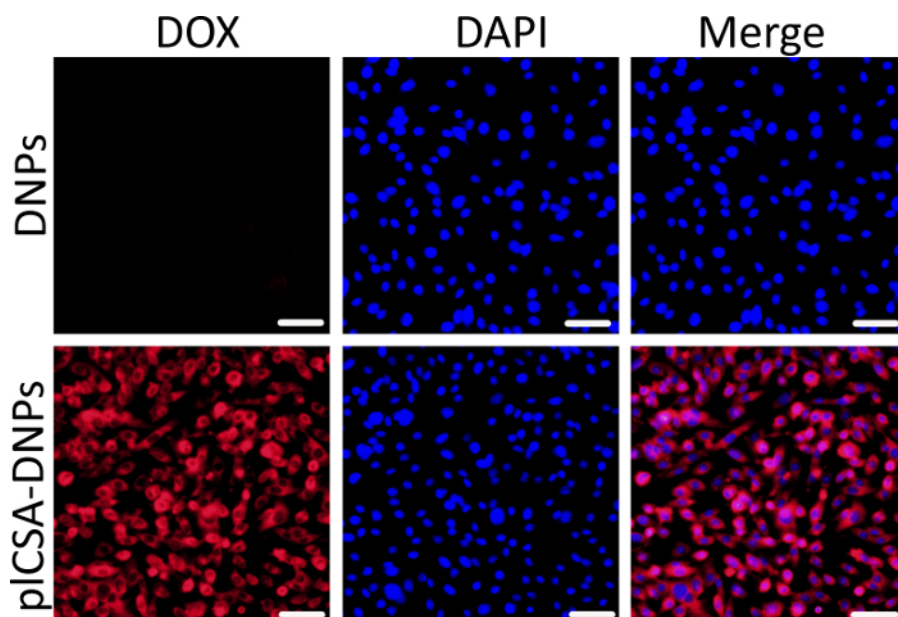


Figure 5. Nanoparticle uptake by JEG3 cells. JEG3 cells were analyzed by fluorescence microscopy after 30 min of incubation with 5 µg/mL different nanoparticles. Red: DOX, blue: DAPI-labeled nuclei. Scale bar=50 µm. [Please click here to view a larger version of this figure.](#)

Discussion

This protocol provides an efficient and reproducible method for synthesizing pICSA-BP-conjugated lipid-polymer nanoparticles. The single-step sonication method to prepare lipid-polymer nanoparticles is fast, reproducible and different from typical nanoprecipitation methods that involve heating, vortexing, or evaporation. Hence, the developed method significantly reduces the synthesis time. In addition, the EDC/NHS bioconjugate used in this protocol is a commonly used and convenient technique to conjugate peptides and antibodies to nanoparticles. Therefore, the combination of the single-step sonication method with the EDC/NHS bioconjugate technique to synthesize pICSA-targeting lipid-polymer nanoparticles can be scaled up for clinical applications.

Certain procedures are critical for successfully synthesizing and characterizing pICSA-DNPs. First, the relative concentrations of lecithin, PLGA and DSPE-PEG-COOH determine the lipid-polymer nanoparticle size and polydispersity. When the concentration of lecithin is fixed, a higher

concentration of DSPE-PEG-COOH results in lower polydispersity and a smaller nanoparticle size,^{13,15} which consequently decreases the drug LE. When the concentration of PLGA is fixed, more DSPE-PEG-COOH is replaced by lecithin, leading to higher nanoparticle polydispersity and larger nanoparticle size¹⁵. In this situation, the nanoparticles are more unstable and aggregate easily. In this protocol, the DSPE-PEG-COOH/PLGA weight ratio is 0.11, and the lecithin/DSPE-PEG-COOH mass ratio is 0.43. The lipid-polymer nanoparticle size is approximately 82 nm with a PDI of approximately 0.127.

Second, the pH of the solution during the conjugation of the peptides to the nanoparticles is of utmost importance. The optimal pH of ester activation is 5.0-6.0, and the amine reaction is most efficient at pH 7.2-7.5^{19,28,29}. For best results, the reaction should be performed in two steps. First, ester activation occurs in MES (or another noncarboxylate, nonamine buffer) at pH 5.0-6.0. Then, the pH is adjusted to 7.2-7.5 using PBS (or another nonamine buffer) immediately before the reaction with the amine-containing molecule¹⁹. Considering that the half-life of NHS esters is 4-5 h at pH 7.0¹⁹, the duration of the amine reaction exceeds 10 h at 4 °C.

The final critical factor is nanoparticle uptake. Nontargeted uptake of DNPs by JGE3 cells was eliminated in a function analysis of the targeted uptake by controlling the culture time and temperature and the concentration of FBS in the medium. In this protocol, JEG3 cells were incubated with 5% FBS at 4 °C for 1 h, and then cultured at 37 °C for 30 min to minimize DNP cellular uptake.

If issues arise during the procedure, there are three major troubleshooting steps related to the synthesis and characterization of pICSA-DNPs. First, lipid-polymer nanoparticles were prepared by the self-assembly of PLGA and lecithin under sonication conditions. PLGA only dissolves in organic solvent. Hence, upon addition to a hydrophilic solvent, PLGA precipitates quickly into small nanoparticles. To minimize the effect of the speed of the dropwise addition and avoid generating larger particles, sonication of the mixture for 1-2 min before adding PLGA is necessary. Second, although quantitative analysis of the pICSA-BP conjugation efficiency using the BCA method is quick and convenient, a high-performance liquid chromatography (HPLC) assay is more accurate. The HPLC experimental details have been described by our group¹¹ and others^{30,31,32}. Finally, the optimal conditions for pICSA-DNP cellular uptake may differ for other cancer cells. Gradually decreasing the FBS concentration and increasing incubation time at 37 °C may help determine the optimal conditions.

One limitation of the protocol is that the hydrophobicity of drugs determines the EE of the drugs. Compared with hydrophilic drugs, hydrophobic drugs have a stronger affinity for PLGA, which results in an increased drug EE. In these instances, the procedures for the synthesis of the pICSA-targeted lipid-polymer nanoparticles will have to be examined experimentally to determine the most efficient synthesis method that results in the ideal drug LE and EE.

As mentioned above, the glycosaminoglycan pICSA is specifically expressed on most cancer cells and placental trophoblasts. Hence, it is important to note that the pICSA-NPs should be used for the targeted delivery of therapeutics to tumor cells in only nonpregnant animals and humans, and that these drugs-loaded particles would lead to side effects in the placenta of pregnant animals and humans.

In summary, we have described a simple and convenient method to synthesize pICSA-targeted lipid-polymer nanoparticles. The significance of this protocol is that it increases the drug availability at tumors and the placenta, while minimizing the concomitant toxicity. This approach should be useful for the targeted delivery of drugs to tumors and placenta and may be scaled up to prepare pICSA-targeted lipid-polymer nanoparticles for clinical applications in the future.

Disclosures

X.F. and B.Z. are the inventors on the patent PCT/CN2017/108646 and 201710906587.6 submitted by SIAT that covers a pICSA-targeted nanoparticle synthesis method and application. No potential conflicts of interest were disclosed by the other authors.

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