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Engineering of a tripeptide-stabilized nanoemulsion of oleic acids for biological imaging and drug delivery applications

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October 8, 2018

Dear Dr. Steindel,

Please find enclosed our manuscript entitled “Engineering of a tripeptide-stabilized nanoemulsion of oleic acid for biological imaging and drug delivery applications,” which we would like to submit for publication in JOVE *Bioengineering*.

We implemented all editorial changes in the protocol as well as reviewers requests. The new and modified text is in red. We thank the reviewers for their valuable criticism. Should you have any additional questions please do not hesitate to contact us.

Sincerely yours,
Aneta Mieszawska, PhD

TITLE:

A Tripeptide-Stabilized Nanoemulsion of Oleic Acid

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KEYWORDS:

Nanoemulsion, oleic acid, tripeptide, anticancer therapy, biological imaging, drug delivery

SUMMARY:

This protocol describes an efficient method to synthesize a nanoemulsion of an oleic acids–platinum(II) conjugate stabilized with a lysine-tyrosine-phenylalanine (KYF) tripeptide. The nanoemulsion forms under mild synthetic conditions via self-assembly of the KYF and the conjugate.

ABSTRACT:

We describe a method to produce a nanoemulsion composed of an oleic acids–Pt(II) core and a lysine-tyrosine-phenylalanine (KYF) coating (KYF-Pt-NE). The KYF-Pt-NE encapsulates Pt(II) at 10 wt. %, has a diameter of 107 ± 27 nm and a negative surface charge. The KYF-Pt-NE is stable in water and in serum, and is biologically active. The conjugation of a fluorophore to KYF allows the synthesis of a fluorescent nanoemulsion that is suitable for biological imaging. The synthesis of the nanoemulsion is performed in an aqueous environment, and the KYF-Pt-NE forms via self-assembly of a short KYF peptide and an oleic acids–platinum(II) conjugate. The self-assembly process depends on the temperature of the solution, the molar ratio of the substrates, and the flow rate of the substrate addition. Crucial steps include maintaining the optimal stirring rate during the synthesis, permitting sufficient time for self-assembly, and pre-concentrating the nanoemulsion gradually in a centrifugal concentrator.

INTRODUCTION:

In recent years, there has been a growing interest in the engineering of nanoparticles for such biomedical applications as drug delivery and bioimaging[1-4]. The multifunctionality of nanoparticle-based systems often necessitates incorporating multiple components within one formulation. The building blocks that are based on lipids or polymers often differ in terms of their physicochemical properties as well as their biocompatibility and biodegradability, which ultimately might affect the function of the nanostructure[1,5-6]. Biologically derived materials, such as proteins and peptides, have long been recognized as promising components of multifunctional nanostructures due to their sequence flexibility[7-8]. Peptides self-assemble into highly ordered supramolecular architectures forming helical ribbons[9-10], fibrous scaffolds[11-12], and many more, thus paving the way to building biomolecule-based hybrid nanostructures using a bottom-up approach[13].

Peptides have been explored for applications in medicine and biotechnology, especially for anticancer therapy[14] and cardiovascular diseases[15] as well as for antibiotic development[16-17], metabolic disorders[18], and infections[19]. There are over a hundred of small-peptide therapeutics undergoing clinical trials[20]. Peptides are easy to modify and fast to synthesize at low cost. In addition, they are biodegradable, which greatly facilitates their biological and pharmaceutical applications[21-22]. The use of peptides as structural components includes the engineering of responsive, peptide-based nanoparticles and hydrogel depots for controlled release[23-27], peptide-based biosensors[28-31], or bio-electronic devices[32-34]. Importantly, even short peptides with two or three amino-acid residues that include phenylalanine were found to guide the self-assembly processes[35-37] and create stabilized emulsions[38].

Platinum-based drugs, owing to their high efficacy, are used in many cancer treatment regimens, both alone and in combination with other agents[39-40]. Platinum compounds induce DNA damage by forming monoadducts and intrastrand or interstrand cross-links. The Pt-DNA lesions are recognized by the cellular machinery and, if not repaired, lead to cellular apoptosis. The most important mechanism, by which Pt(II) contributes to cancer cell death, is the inhibition of DNA transcription[41-42]. However, the benefits of platinum therapy are diminished by systemic toxicity of Pt(II) that triggers severe side effects. This leads to lower clinical dosing of Pt(II)[43], which often results in sub-therapeutic concentrations of platinum reaching the DNA. As a consequence, the DNA repair that follows contributes to cancer cell survival and acquiring Pt(II) resistance. The platinum chemo-resistance is a major problem in anticancer therapy and the main cause of treatment failure[44-45].

We have developed a stable nanosystem that encapsulates the Pt(II) agent in order to provide a shielding effect in systemic circulation and to diminish the Pt(II)-induced side effects. The system is based on an oleic acids–Pt(II) core stabilized with a KYF tripeptide to form a nanoemulsion (KYF-Pt-NE)[46]. The building blocks of KYF-Pt-NE, the amino acids of the tripeptide as well as the oleic acid, have the Generally Recognized As Safe (GRAS) status with Food and Drug Administration (FDA). The KYF-Pt-NE is prepared by using a nanoprecipitation method[47]. In short, the oleic acids–Pt(II) conjugate is dissolved in an organic solvent and then added dropwise to an aqueous KYF solution (**Figure 1**) at 37 °C. The solution is stirred for several hours to allow self-assembly of the KYF-Pt-NE. The nanoemulsion is concentrated in 10 kDa centrifugal concentrators and washed

three times with water. The chemical modification of the KYF with a fluorophore allows the synthesis of fluorescent FITC-KYF-Pt-NE suitable for biomedical imaging.

PROTOCOL:

1. Synthesis of the Oleic Acids–Platinum(II) Conjugate

1.1. Activation of cisplatin

1.1.1. Suspend 50 mg (0.167 mmol) of cisplatin in 4 mL of water (e.g., nanopure) at 60 °C.

1.1.2. Add dropwise 55.2 mg (0.325 mmol) of $AgNO_3$ in 0.5 mL of water to the solution of cisplatin and stir the reaction for at least 2 h at 60 °C. The white precipitate of AgCl will form indicating the progress of the reaction.

1.1.3. To determine if the activation reaction is completed, perform the test with 10% HCl for the presence of free Ag^+ ion in solution. The test should be negative (no additional AgCl precipitate should form).

1.1.4. Centrifuge the reaction mixture at 3,220 x *g* for 10 min and remove the white precipitate of AgCl.

1.1.5. Collect the supernatant and filter it via a 0.2 μm syringe filter. Test the supernatant for the presence of platinum by applying 2-3 drops of the solution via Pasteur pipette to $SnCl_2$ crystals. The test is positive if the color of coordinate complex of tin with platinum is dark yellow/orange.

1.1.6. Use the supernatant with activated Pt(II) for the second step.

1.2. Reaction of oleic acid with activated Pt(II)

1.2.1. Dissolve 13.3 mg (0.333 mmol) of NaOH in 3 mL of water at 60 °C to obtain a 0.11 M solution.

1.2.2. Add 94.2 mg of oleic acid (0.333 mmol) to 0.11 M NaOH, and mix with the solution of activated Pt(II) from step 1.1.

1.2.3. Stir the reaction for 2 h at 60 °C and next at room temperature overnight. The crude product is an oily brown/yellow precipitate.

1.2.4. Centrifuge the reaction mixture at 3,220 x *g* for 10 min and remove the supernatant. Dry the crude product at 25 °C using a rotary evaporator. Purify the product by multiple washes with acetonitrile. The final color of pure oleic acids–Pt(II) conjugate is pale yellow.

2. Synthesis of KYF-Pt-NE, and the FITC-labeled Nanoemulsion FITC-KYF-Pt-NE

2.1. Synthesis of the KYF tripeptide and the fluorescently labeled tripeptide FITC-KYF

2.1.1. Synthesize the KYF using standard solid-state peptide chemistry. Use the following standard coupling conditions to attach each amino acid: Wang resin (2.19 mmol), Fmoc protected amino acid (4.38 mmol), 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethylaminium tetrafluoroborate (TBTU) (4.38 mmol) and diisopropylethylamine (DIPEA) (8.76 mmol). Dissolve the amino acids with TBTU in dimethylformamide (DMF) and DIPEA.

2.1.2. Soak 5.7 g of Fmoc-L-Phe 4-alkoxybenzyl alcohol resin (0.382 meq/g) in 25 mL of DMF for 1 h prior to use.

2.1.3. Deprotect the amine group of L-phenylalanine amino acid with 15 mL of 20% piperidine/DMF solution for 5 min, discard the solvent and repeat the wash with 20 min cycle.

2.1.4. Wash the resin for 1 min with the following solvents: DMF, isopropyl alcohol (IPA), DMF, IPA, DMF, IPA, DMF, DMF. Discard the solvent after each wash.

2.1.5. Perform the Kaiser test to determine the presence of free NH_2 group on the resin (see substeps) and if positive (the resin seed is purple), add the Fmoc-L-tyrosine amino acid and perform the coupling overnight.

2.1.5.1. Prepare Kaiser test solutions in separate bottles.

2.1.5.2. Dissolve 5 g of ninhydrin in 100 mL of ethanol.

2.1.5.3. Dissolve 80 g of phenol in 20 mL of ethanol.

2.1.5.4. Mix 2 mL of 0.001 M aqueous solution of potassium cyanide with 98 mL of pyridine.

2.1.5.5. Add 2-3 drops of each Kaiser test solutions to sample and heat in boiling water for 5 min.

2.1.6. Upon successful coupling of second amino acid, perform the Kaiser test and if negative proceed with deprotection protocol (steps 2.1.3 to 2.1.5). Repeat the process with the Fmoc-phenylalanine amino acid.

2.1.6.1. Upon coupling of all amino acids, wash the resin for 1 min with 5 mL of DMF, IPA, DMF, methanol, dichloromethane and diethyl ether, after each wash discard the solvent. Save the resin for further processing.

2.1.6.2. Use half of the resin for the next steps (2.1.7-2.1.9) to modify the peptide with FITC. To obtain unmodified KYF tripeptide, follow the procedure starting at 2.1.10.

2.1.7. Modify the N-terminal amino acid of the KYF to KYF-N_3 with 6-azidohexanoic acid. To this end, mix 1 g (0.382 mmol) of KYF Wang resin and 120.1 mg (0.764 mmol) of 6-azidohexanoic acid with 245.2 mg (0.764 mmol) of TBTU and 197.1 mg (1.528 mmol) of DIPEA in 30 mL of DMF. Stir the reaction overnight at room temperature.

2.1.8. Obtain the KYF-FITC from the synthesis of the KYF-N_3 with propargyl fluorescein via a click reaction. To this end, mix 253 mg (0.097 mmol) of the KYF-N_3 Wang resin with 3.78 mg (0.019 mmol) of CuI solid, 71.9 mg (0.193 mmol) of propargyl fluorescein, and 2.24 mg (0.017 mmol) of DIPEA. The reaction should change color from green to brown.

2.1.9. After 24 h, wash the resin for 1 min alternately with 5 mL of DMF and IPA five times, methanol and water thrice, DMF and water thrice, and with dichloromethane and diethyl ether thrice. Discard the solvent after each wash.

2.1.10. Cleave the KYF-FITC or KYF peptide from the resin with a solution of trifluoroacetic acid (TFA)/triisopropylsilane (TIPS)/H_2O at the ratio of 95/2.5/2.5 over 3 hours.

2.1.11. Precipitate the crude peptide in a cold diethyl ether, wash thrice with cold ether, and then dry under the vacuum.

2.2. Synthesis of FITC-KYF-stabilized nanoemulsion with Pt(II) (FITC-KYF-Pt-NE) and KYF-Pt-NE

2.2.1. Dissolve 10 mg (0.0126 mmol) of oleic acids–Pt(II) conjugate in 1.5 mL of isopropanol and place in a 5 mL syringe.

2.2.2. Place the syringe with oleic acids–Pt(II) conjugate in a syringe pump and set the flow to 0.1 mL/min.

2.2.3. In order to synthesize the FITC-KYF-Pt-NE, dissolve 1 mg (0.00105 mmol) of FITC-KYF and 1 mg (0.00219 mmol) of KYF (1:2 molar ratio of FITC-KYF:KYF) in 20 mL of water and adjust the temperature of the solution to 37 °C. Cover the walls of the container with aluminum foil to avoid photobleaching of the FITC fluorophore. To synthesize the KYF-Pt-NE, dissolve 2 mg (0.0044 mmol) of KYF tripeptide in 20 mL of water and adjust the temperature of the solution to 37 °C.

2.2.4. Add the oleic acids–Pt(II) conjugate dropwise to the solution of FITC-KYF/KYF or KYF tripeptide. Perform this step under the hood.

2.2.5. Stir the solution overnight at room temperature to evaporate organic solvents and to allow the self-assembly of FITC-KYF-Pt-NE or KYF-Pt-NE.

2.2.6. Concentrate the FITC-KYF-Pt-NE or KYF-Pt-NE in a centrifugal concentrator (10k MWCO), and wash thrice with 4 mL of the nanopure water.

2.2.7. Store the aqueous solutions of KYF-Pt-NE and FITC-KYF-Pt-NE at 4 °C.

2.2.8. Analyze for platinum content using atomic absorption spectroscopy (AAS), following the manufacturer's guide[48].

2.2.8.1. Prepare platinum standards in 10% HCl solution for the calibration curve (effective range for AAS is between 100 to 1200 ppb (parts per billion)).

2.2.8.2. Dissolve 50 µL of KYF-Pt-NE solution from step 2.2 in 100 µL of aqua regia (a mixture 3:1 of concentrated hydrochloric acid and nitric acid) and leave at room temperature overnight. Add 850 µL of water to reach a final sample volume of 1 mL. Analyze the sample using AAS. The final acid concentration should be 10% in all analyzed samples.

2.2.8.3. Record the reading of Pt concentration in ppb, and compute the final platinum content in the sample (account for sample dilution and initial volume of nanoemulsion).

3. Confocal Imaging of the Cellular Uptake of FITC-KYF-Pt-NE

3.1. Seed 6 ovarian cancer cell lines (A2780, CP70, SKOV3, OV90, TOV21G, and ES2), into 4 well-chamber confocal dishes at the density of 4.7 x 10^4 cells per chamber, and pre-culture overnight at 37 °C.

3.2. After 24 h, wash the cells thrice with phosphate buffer saline (PBS) and incubate with FITC-KYF-Pt-NE in cell culture medium (see step 6.1 for cell line specific details) for 15 min at 37 °C.

3.3. After incubation, remove the media and wash the cells three times with PBS.

3.4. Fix the cells with cold methanol for 5 min at -20 °C and wash three times with 1 mL of PBS.

3.5. Permeabilize the cells with 1 mL of 0.1% Triton-X for 10 min at room temperature and wash three times with 1 mL of PBS.

3.6. Incubate the cells for 90 min with LAMP1 antibody conjugated with Alexa Fluor 647 (1 mL) at 1:50 dilution in PBS at room temperature. Next, wash the cells 3 times with PBS.

3.7. Dilute DAPI stock solution (1 mg/mL) to 1 µg/mL in PBS. Then, add 1 mL of the diluted solution to each chamber and incubate for 15 min at room temperature. Wash the cells 3 times with PBS.

3.8. Mount coverslips on a slide using mounting medium.

3.9. Image the cells using live cell confocal microscope at excitation wavelength of 405 nm, 488 nm and 633 nm. Set the detection parameters as follow: laser power from 0.2% and no more than 1%, Pinole 1 Airy unit, Gain master 650-750, Digital offset 0.

3.10. Open image in imaging software. Under displayed image, select **Graphics** and select **Insert Scale Bar**, to insert the scale bar to the image.

4. Drug Release Studies

4.1. Carry out the drug release studies in PBS. Adjust the pH values of three PBS buffers to 7.4, 6.8 and 5.0 respectively.

4.2. Dilute 5 µL of KYF-Pt-NE in 180 µL of appropriate pH PBS buffer, transfer to 3.5 kDa MWCO mini dialysis cup and incubate at 37 °C in PBS.

4.3. Remove buffer from each three mini dialysis tubes at 2, 4, 6, 24, 48, and 140 h, and measure the platinum concentrations by AAS. Prepare all samples according to step 2.2.8 but adjust the final volume of the sample to 500 µL.

5. Cell Culture Methods

5.1. Culture cell lines A2780, CP70, SKOV-3, OV-90, TOV-21G, ES-2 in cell culture medium (DMEM) supplemented with 10% (A2780, CP70, SKOV-3, ES-2) or with 15% (TOV-21G, OV-90) fetal bovine serum (FBS), with L-Glutamine and penicillin/streptomycin. Grow all cells in a 5% CO_2, water saturated atmosphere at 37 °C.

5.2. Seed 3 x 10^5 cells in each 96-well plate and pre-culture overnight for in vitro incubation experiments. Prepare the KYF-Pt-NE, cisplatin, and carboplatin solutions in water. Adjust the concentration of Pt(II) in KYF-Pt-NE to match the concentration of carboplatin and cisplatin for each cell line. Incubate for 72 hours at 37 °C.

5.3. After incubation, evaluate the cellular viability using a colorimetric assay (the MTT Cell Proliferation assay). Briefly, remove medium and add 110 µL of 10% MTT in medium to each well and incubate for 2 h at 37 °C. Then add 100 µL detergent to each well and incubate for 5 h at 37 °C.

5.4. Check the absorbance at 570 nm by using plate reader. Analyze results using statistical analysis with Z-test and P-test.

REPRESENTATIVE RESULTS:

Representative TEM image of KYF-Pt-NE prepared using this protocol is shown in **Figure 2A**. The KYF-Pt-NEs are spherical in morphology, well dispersed, and uniform in size. The core diameter of the KYF-Pt-NEs, measured directly from three TEM images with a minimum of 200 measurements done, is 107 ± 27 nm. The hydrodynamic diameter of KYF-Pt-NE, analyzed using dynamic light spectroscopy (DLS), was found to be 240 nm with a polydispersity index of 0.156. The Zeta potential of KYF-Pt-NE in water was determined for three independent syntheses with the average value of -60.1 mV. The high magnitude of the potential indicates good colloidal

stability of the formulation, and the negative surface charge is attributed to ionized COO^- surface groups of oleic acids. The isoelectric point of KYF is 8.59, and therefore it is positively charged at neutral pH.

The ability of the nanoemulsions to cross the cellular membrane was examined using the fluorescently labeled FITC-KYF-Pt-NE and ovarian cancer cell lines. The results are presented in **Figure 2B**. It can be clearly seen that the FITC-KYF-Pt-NEs (green) are distributed within the cytosol but were not yet associated with lysosomes (red). This result demonstrates that KYF-Pt-NEs enter cells and can serve as intracellular drug delivery vehicle.

The stability of KYF-Pt-NEs and FITC-KYF-Pt-NEs was assessed with DLS. The diameter of the nanoemulsions in water was measured over several months and the results are presented in **Figure 3**. The hydrodynamic diameter of both formulations slowly increased during 4 months in storage, to 320 nm (KYF-Pt-NE) and 240 nm (FITC-KYF-Pt-NE), but the nanoscale dimensions were preserved. This result suggests that the KYF tripeptide effectively stabilizes nanoemulsions over long periods of time.

The Pt(II) encapsulation efficiency and release from the nanoemulsion were measured with AAS. The Pt(II) concentration in KYF-Pt-NE was established to be 10 wt.%. The Pt(II) release from KYF-Pt-NE at pH 7.4 (physiological), 6.8 (tumor's interstitium)[49], and 5.0 (endosomal)[50] is presented in **Figure 4A**. The Pt(II) release is the slowest at pH 7.4 with only 20.8% of Pt(II) released after 4 h, while at pH 6.8 and 5.0 the release was 32.8% of and 47.5%, respectively. The same trend continued after 24 h and after 6 days. This result indicates that Pt(II) release from KYF-Pt-NE is pH dependent, and it can be delayed in the systemic circulation, and accelerated once the nanoemulsions translocate into tumor.

The biological activity of the KYF-Pt-NE was established in vitro using the same ovarian cancer cell lines as in the imaging studies. The cells were incubated with KYF-Pt-NE for 72 h, at KYF-Pt-NE concentrations corresponding to IC_{50} for each cell line. The viability was assessed using the MTT assay and the results were compared to cells only, KYF-NE, oleic acids–Pt(II) conjugate, carboplatin, and cisplatin. The results of biological activity of KYF-Pt-NE are shown in **Figure 4B**. The KYF-Pt-NE reduced the viability of isogenic cell lines A2780 (Pt sensitive) and CP70 (Pt resistant) by 44.3% and 46.2% respectively. Carboplatin, the clinically relevant analogue, decreased the viability by 18.5% (A2780) and 9.6% (CP70) only. The same trend of greater KYF-Pt-NE effect on cell death was observed across other cell lines as well. The viability of Pt(II) sensitive TOV-21G cells was reduced by 55.9% after incubation with KYF-Pt-NE, while carboplatin resulted in lowering the viability by just 16.5%. In OV-90 cells with intermediate Pt(II) resistance, the viability was lowered by 55.3% (KYF-Pt-NE) and 23.9% (carboplatin). The two resistant cancer cell lines, ES-2 and SKOV-3, showed reduction in viability by 45.9% and 54.3%, respectively, for KYF-Pt-NE, and 10.3% (ES-2) and 16.8% (SKOV-3) for carboplatin.

The biological activity of KYF-Pt-NE versus cisplatin was also compared. Cisplatin is the Pt(II)-based agent of first generation that is no longer favored in the clinic due to its toxicity profile[51]. In two cell lines, SKOV-3 and TOV-21G, the viability reduction was greater by 15% and 40%,

respectively, for KYF-Pt-NE than for cisplatin. In the remaining cell lines, the activity of KYF-Pt-NE was comparable to cisplatin (A2780, CP70, OV-90), or slightly lower (ES-2). The oleic acids–Pt(II) conjugate was also found to be biologically active. However, KYF-Pt-NE showed higher reduction in viability than the conjugate in majority of the cell lines tested, indicating the significance of nanoformulation in Pt(II) activity.

The biological applications of nanoparticulate systems require stability in biologically relevant media, thus the stability of KYF-Pt-NE was evaluated in 20% fetal bovine serum (FBS) and the results are presented in **Figure 4C**. We detected no evidence of KYF-Pt-NE opsonization in serum after one day of incubation.

Figure Legends:

Figure 1. The components of the nanoemulsions (top) and schematic of the nanoemulsion preparation (bottom).

Figure 2. TEM image of KYF-Pt-NE (A) and confocal microscope images of FITC-KYF-Pt-NE (green) uptake by ovarian cancer cells (nuclei are blue and lysosomes are red) (**B**). Scale bars are 10 μm. This Figure has been adapted with permission from *Bioconjugate Chemistry* 2018, 29, 2514-2519.[46] Copyright 2018 American Chemical Society.

Figure 3. Stability of KYF-Pt-NE and FITC-KYF-Pt-NE in water. Each point represents the mean and standard deviation of N=3 and $p < 0.005$. This Figure has been adapted with permission from *Bioconjugate Chemistry* 2018, 29, 2514-2519.[46] Copyright 2018 American Chemical Society.

Figure 4. KYF-Pt-NE stability and biological activity. (**A**) Pt(II) release from KYF-Pt-NE in PBS buffer at different pHs (PBS, 37 °C). Cellular viability of different ovarian cancer cell lines after 72 h incubation with KYF-Pt-NE (**B**). Each column represents the mean and standard deviation of N=3 and $p < 0.005$. The concentrations are constant in each cell line and are as follows: 4.92 μM (A2780), 8.80 μM (CP70), 2.46 μM (TOV-21G), 9.84 μM (SKOV3), 7.38 μM (ES-2), 19.7 μM (OV-90). The concentration of KYF-Pt-NE and oleic acids–Pt(II) conjugate was adjusted with respect to Pt(II) content measured by AAS. Abbreviations: "Carbpt" – carboplatin; "KYF-NE" – KYF tripeptide-coated nanoemulsion; "KYF-Pt-NE" – KYF tripeptide-coated nanoemulsion containing Pt(II); Cispt – cisplatin; Pt-oleic – oleic acids–Pt(II) conjugate. (**C**) KYF-Pt-NE stability in serum. This Figure has been adapted with permission from *Bioconjugate Chemistry* 2018, 29, 2514-2519.[46] Copyright 2018 American Chemical Society.

DISCUSSION:

Critical steps in the nanoemulsion synthesis include adjusting the molar ratio of the substrates, maintaining temperature and flow rate control during oleic acids–Pt(II) addition, providing sufficient time for self-assembly, and purifying the product using a centrifugal concentrator column. These parameters influence the size and morphology of the KYF-Pt-NE; thus, it is particularly important to maintain the proper molar ratio and adjust the synthetic conditions correctly.

The ratio of the substrates during the nanoemulsion synthesis (step 3) is crucial for the self-assembly process and determines the final size of the product. The KYF to oleic acid-Pt(II) molar ratio is 1:3, and the optimal concentration of the KYF tripepide in water is 0.2 mM. In addition, the organic solvent used to dissolve the oleic acid-Pt(II) conjugate in step 3.1 has to be miscible with water, compatible with the filtration system, and evaporative easily at room temperature.

One of the critical steps is the dropwise addition of the oleic acid-Pt(II) conjugate to the KYF solution (step 3.4). The flow should not be slower than 0.1 mL/min and not faster than 0.2 mL/min, because a sub-optimal speed can induce the precipitation of the nanoemulsion. Also, the oleic acids–Pt(II) conjugate should be added to the KYF solution at 37 °C while stirring the mixture on a stir plate at 600 rpm. Once all of the oleic acid-Pt(II) has been added, the solution should be kept at room temperature and the stirring speed reduced to 150 rpm. Step 3.5 should be carried out at room temperature. The optimal time for the self-assembly of the KYF-Pt-NE (step 3.5) is 24 hours.

There is a risk that the product may precipitate while the nanoemulsion is being pre-concentrated. Therefore, it is recommended that the nanoemulsion be diluted with additional water before being centrifuged in a centrifugal concentrator and spun no faster than 2,465 x *g*. The diluted nanoemulsions should be added to the centrifugal concentrator at aliquots between spins, and the nanoemulsion should be mixed in the filter before the next portion is added.

The ability to form nanoemulsions with fatty acids derivatives other than oleic acid has not been tested and is yet to be determined. Future applications may include the use of different peptides to facilitate the co-assembly with oleic acid. Also, oleic acid conjugates with non-platinum-based drugs may be used as potential cores of nanoemulsions.

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DISCLOSURES:
Authors do not have a conflict of interest to disclose.

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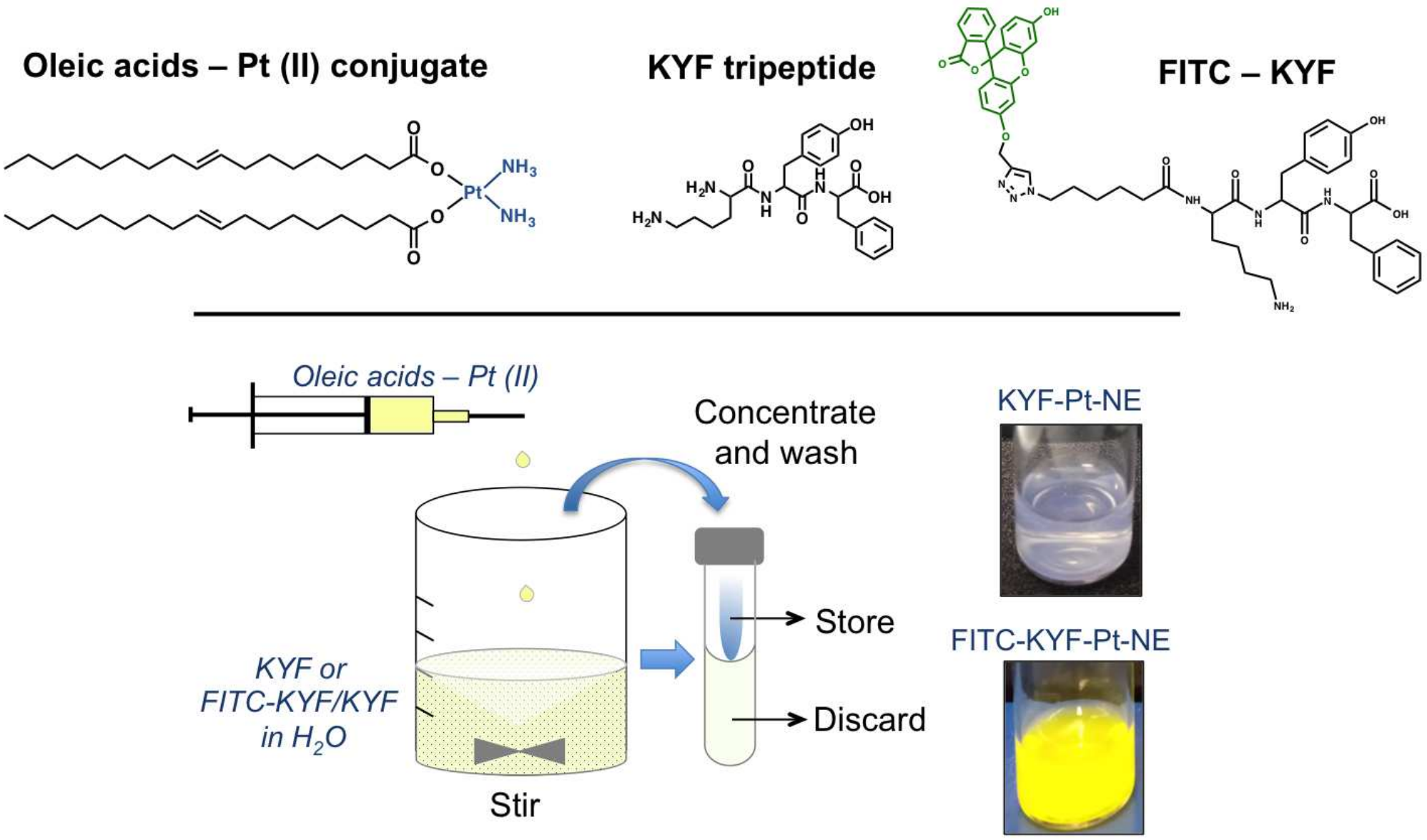

Figure

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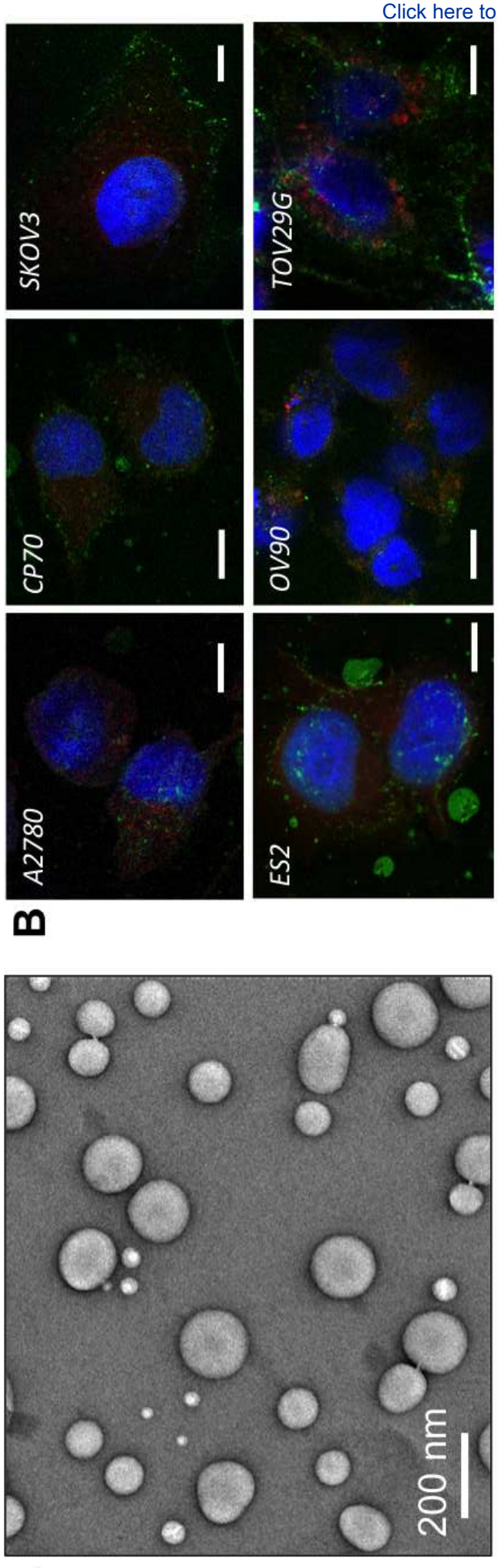

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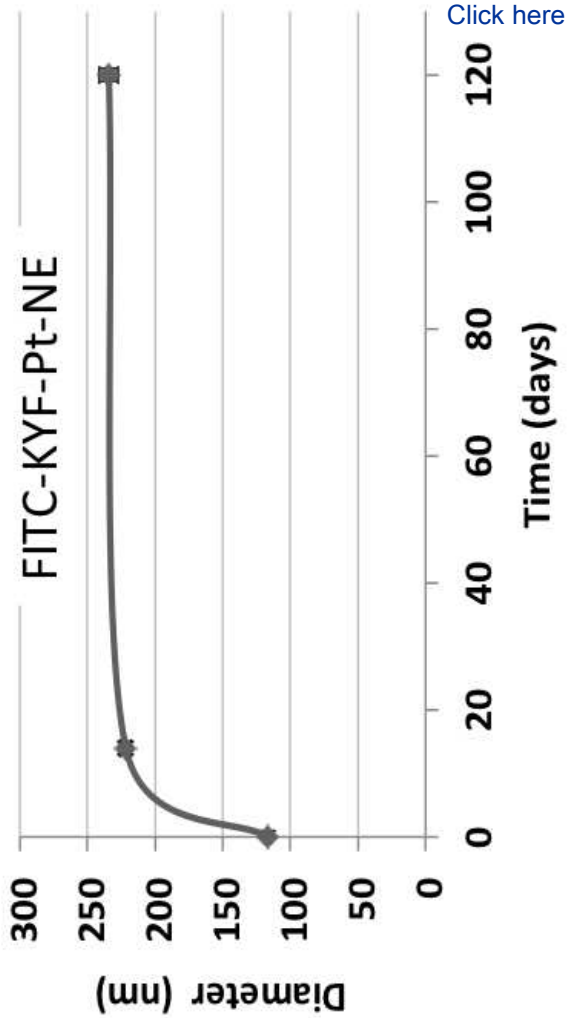

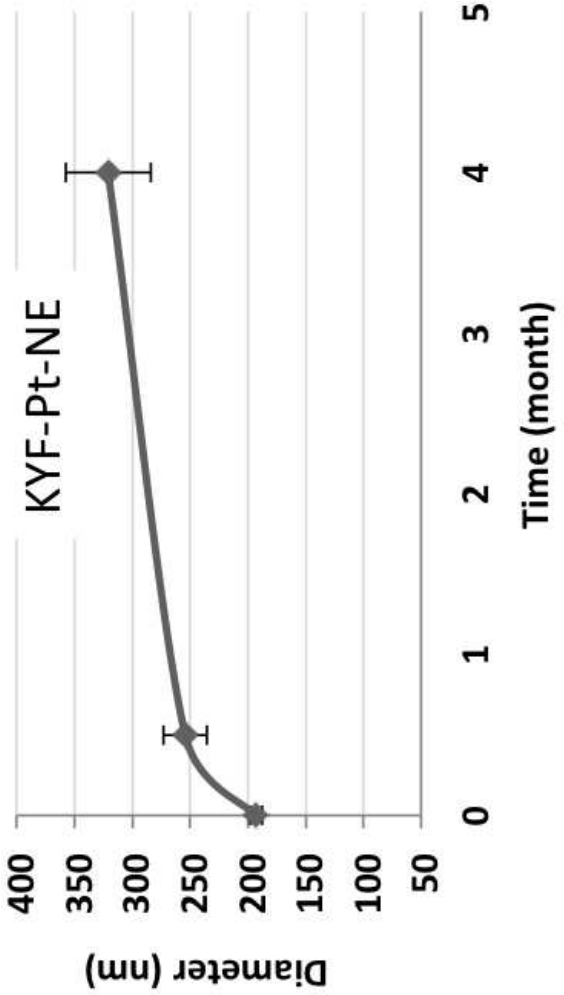

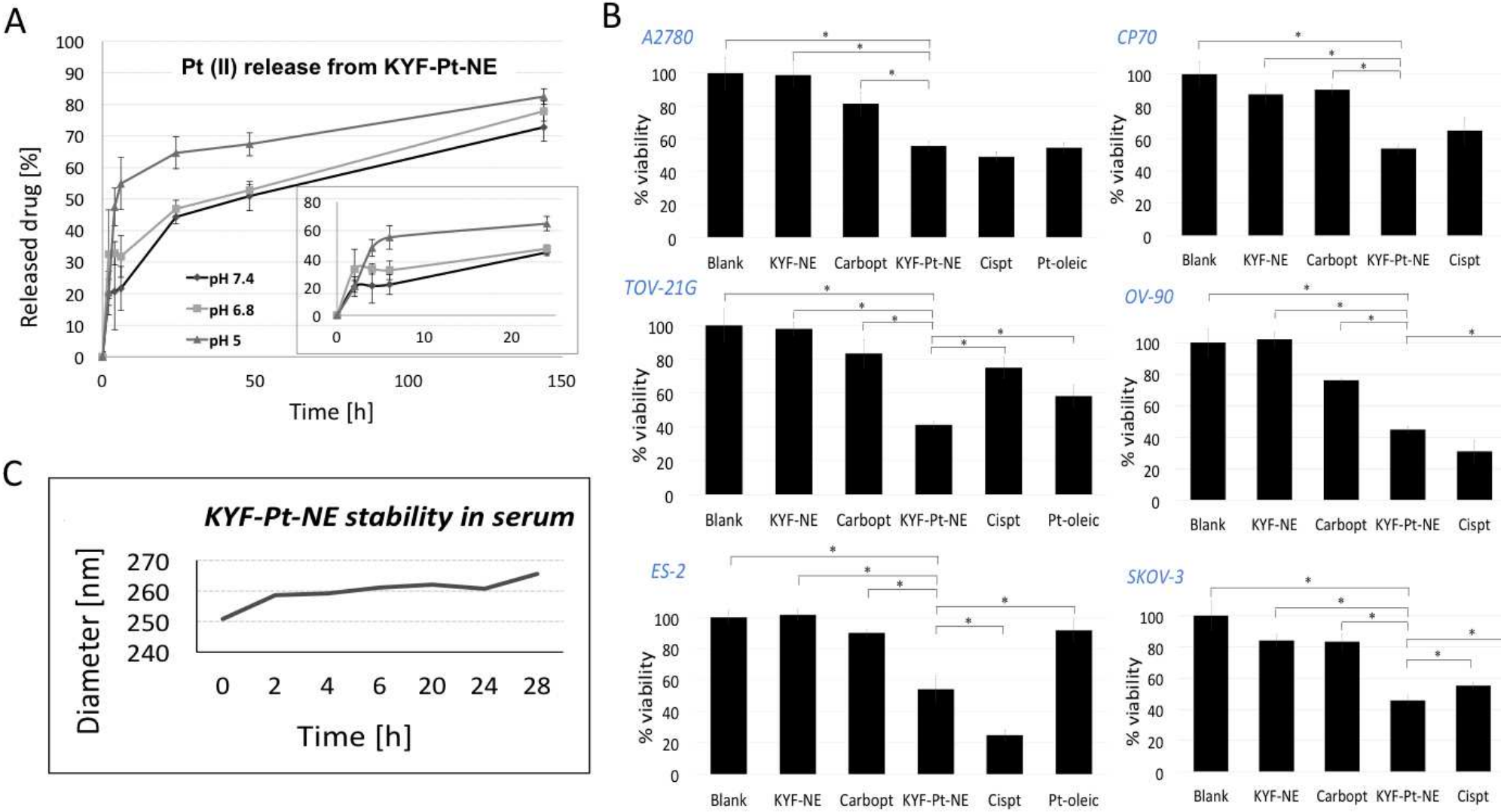

Name of Reagent/ Equipment	Company	Catalog Number
2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU)	ANASPEC INC.:	AS-20376
4-well chamber confocal dish	Lab-Tek II, Thermo Fisher Scientific	154526
6-bromohexanoic acid	Chem-Impex INT'L INC.	24477
A2780	Generously doanted by professor John Martignetti from The Mount Sinai Hospital	
Barnstead Nanopure	Thermo Fisher	D11901
BUCHI rotavapor R-3	Buchi	Z568090
Centrifuge 5810 R	eppendorf	5811F
Cis-dichlorodiamineplatinum (II) 99%	Acros Organics	19376-0050
CP70	Generously doanted by professor John Martignetti from The Mount Sinai Hospital	
Digital water bath	VWR	97025-134
Dynamic Light Scattering (DLS)	Brookhaven Instrument Corporation	
ES-2	ATCC	CRL-1978
Fmoc-L-Lys(Boc)-OH 99.79%	Chem-Impex INT'L INC.	00493
Fmoc-L-Phe 4-alkoxybenzyl alcohol resin (0.382 meq/g),	Chem-Impex INT'L INC.	01914
Fmoc-LTyr(tBu)-OH 98%	Alfa Aesar	H59730
HERACELL 150i CO2 incubator	Thermo Scientific Fisher	
High pressure syringe pump	New Era	1010-US
Hotplate/stirrer	VWR	12365-382
LAMP-1 Antibody(cojugated with Alexa Fluor 647)	Santa Cruz Biotechnology	sc-18821 AF647
N,N-diisopropylethylamine (DIPEA)	Oakwood Chemical	005027
Ninhydrin 99%	Alfa Aesar	A10409
Oleic acid	Chem-Impex INT'L INC.	01421
OV90	ATCC	CRL-11732
PBS	Corning	21-031-CV
Permount mounting medium	Fisher Chemical	SP15-100
Phenol	Fisher Chemical	A92500

Phosphotungstic acid	Fisher Chemical	A248-25
Piperidine 99%	BTC	219260-2.5L
Platinum AAS standard soultion	Alfa Aesar	88086
Propargyl bromide 97%	Alfa Aesar	L10595
Scientific biological cabinet	Thermo Scientific Fisher	1385
Self-Cleaning Vacuum System	Welch	2028
Silver nitrate	Acros Organics	19768-0250
SKOV3	ATCC	HTB-77
Sodium hydroxide	Fisher Scientific	S313-1
Tin (II) chloride	Sigma Aldrich	208256
TOV21G	ATCC	CRL-11730
Trifluoroacetic acid 99% (TFA)	Alfa Aesar	L06374
Triisopropylsilane (TIPS)	Chem-Impex INT'L INC.	01966
Triton-X	Sigma Aldrich	T8787-100ML
Uranine powder 40%	Fisher Scientific	S25328A
Vivaspin 20 (10000 MWCO)	Sartorious	VS2001
VWR Inverted Microscope	VWR	89404-462

Comments/Description

SPPS
For imaging
Click modification for peptide

Ovarian cancer cell line
water filtration system
For solvent removal and sample drying
For platinum complex separation
in vitro tests

Ovarian cancer cell line
For warming up media for cell culture

For nanoparticle size measurments
ovarian cancer cell line
SPPS
SPPS
SPPS
incubator
For platinum complex addition in nanoparticle synthesis
For sample stirring and heating
For imaging
SPPS
Kaiser test
For platinum complex synthesis
Ovarian cancer cell line
For cell wash
For imaging
Kaiser test

negative stain for TEM
SPPS
1000ug/ml for calibration curve
For alkyne modification of fluoresceine
Bio-hood for cell culture
Vacuum pump for rotavapor
Cisplatin activation
Ovarian cancer cell line
For platinum complex synthesis
Test for Platinum presence
Ovarian cancer cell line
SPPS
SPPS
For imaging
For alkyne modification of fluoresceine
For Nanoparticle wash and condensation
For cell culture monitoring

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Title of Article: ENGINEERING OF A TRIPEPTIDE-STABILIZED NANOEMULSION OF OLEIC ACID FOR BIOLOGICAL IMAGING AND DRUG DELIVERY APPLICATIO

Author(s): SYLWIA A. DRAGULSKA, MAREK T. WLODARCZYK, MINA POURSHARIFI, ANETA J MIETZAWS

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The new and modified text in the revised manuscript is highlighted in red. We have responded in a point-by-point manner to all comments as detailed below.

Editorial comments:

1. The current highlighted protocol, when formatted according to JoVE guidelines (12 pt Calibri font, spaces between each step/substep, all text aligned to the left margin; see attached) is ~3.5 pages; our limit for filming and video length purposes is 2.75 pages. Please reduce the highlighted portion of the protocol.

The highlighted part of the protocol was reduced.

2. 2-3: This could be reorganized and clarified a bit more; for one, please also mention how the KYF peptide without FITC is prepared/purified. Additionally, 2.2 and 3 are essentially identical, except that KYF-FITC is added in 2.2-perhaps these could be consolidated (this will also reduce the highlighted length).

The parts were reorganized and combined, edited for clarity.

3. 4.1: Where do these cell lines come from? They aren't in the Table of Materials.

The cell lines were added to the Table of Materials.

4. 5.3: Step 2.8 (currently 2.1.8) doesn't seem to have anything to do with prepping samples for AAS; please check. Please also provide a reference for AAS.

The 2.1.8 step was corrected to 2.2. The reference for AAS was added to the protocol.

Title: Tripeptide-Stabilized Oil-in-Water Nanoemulsion of an Oleic Acids–Platinum(II) Conjugate as an Anticancer Nanomedicine

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