Video Article

One Minute, Sub-One-Watt Photothermal Tumor Ablation Using Porphysomes, Intrinsic Multifunctional Nanovesicles

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Abstract

We recently developed porphysomes as intrinsically multifunctional nanovesicles. A photosensitizer, pyropheophorbide α, was conjugated to a phospholipid and then self-assembled to liposome-like spherical vesicles. Due to the extremely high density of porphyrin in the porphyrin-lipid bilayer, porphysomes generated large extinction coefficients, structure-dependent fluorescence self-quenching, and excellent photothermal efficacy. In our formulation, porphysomes were synthesized using high pressure extrusion, and displayed a mean particle size around 120 nm. Twenty-four hr post-intravenous injection of porphysomes, the local temperature of the tumor increased from 30 °C to 62 °C rapidly upon one minute exposure of 750 mW (1.18 W/cm^2), 671 nm laser irradiation. Following the complete thermal ablation of the tumor, eschars formed and healed within 2 weeks, while in the control groups the tumors continued to grow and all reached the defined end point within 3 weeks. These data show how porphysomes can be used as potent photothermal therapy (PTT) agents.

Video Link

The video component of this article can be found at http://www.jove.com/video/50536/

Introduction

Porphysomes are novel multifunctional nanovesicles that we recently developed which are capable of multimodal imaging and therapy[1]. They are formed from self-assembled porphyrin bilayers and contain an extremely high density of porphyrin (over 83, 000/porphysome particle), which generates large extinction coefficient and results in unique structure-dependent fluorescence self-quenching. Porphysomes have good *in vivo* pharmacokinetic and biodistribution properties: they exhibit a blood half-life of 12 hr following systematic administration, and passively accumulate in xenograft tumors with 7.5% ID/g at 24 hr post-injection[2].

Their unique structure and physiochemical properties make porphysome a good candidate for multimodal imaging and image-guided therapy. First of all, containing porphyrin, porphysomes are intrinsically suitable for fluorescence imaging of tumors upon the tumor accumulation[1]. In addition, each porphyrin has a stable site for chelating radioisotopes, therefore, porphysomes can be easily labeled with radioisotopes such as 64Cu for PET imaging[3]. Furthermore, the absorbed light energy is dissipated thermally under laser irradiation exposure when porphysome structure is intact, so porphysomes also exhibit unique photoacoustic imaging and PTT capabilities. It has been shown that 24 hr after intravenous injection of porphysomes, laser irradiation of the porphysome-accumulated tumor induced a rapid temperature increase and strong photothermal tumor ablation. This demonstrated that porphysomes are efficient photothermal enhancers with extinction coefficient as high as gold nanoparticles (AuNPs)[1]. On the other hand, in comparison with other inorganic photothermal agents, including AuNPs, porphysomes show an outstanding advantage in biosafety due to their organic nature. Porphysomes are enzymatically biodegradable and induce minimal acute toxicity in mice with intravenous doses as high as 1,000 mg/kg[1]. Furthermore, similar to liposomes, the large aqueous core of porphysomes could be passively or actively loaded with therapeutic or imaging agents. The optical properties and biocompatibility of porphysomes demonstrate the multimodal potential of organic nanoparticles for biophotonic imaging and therapy.

In this paper, we introduce the synthesis method of pyropheophorbide-lipid conjugates, the manufacturing and the characterization method of porphysomes using high-pressure extrusion. *In vivo* PTT on mice is conducted as well to demonstrate the efficiency of porphysome-enabled PTT in the tumor treatment using a subcutaneous xenograft tumor model.

Protocol

1. Synthesis of Pyropheophorbide-lipid

1. Combine 200 mmol pyropheophorbide (prepared from *Spirulina Pacifica* algae as described previously; Zheng *et al.*, Bioconj. Chem., 2002, *13*-392)[4], 98.7 mg 16:0 lysophophatidylcholine (1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine, Avanti Polar Lipids #855675), 76.3 mg of EDC , 48.7 mg DMAP (4-(dimethylamino)pyridine) in 5 ml amylene stabilized chloroform for a ratio of 1:1:2:2 Pyro:Lipid:EDC:DMPA.
2. Stir the reaction mixture at room temperature under argon for 24 hr. Evaporate the chloroform under reduced pressure using a rotary evaporator with heating to 40 °C. The mixture of pyro-lipid isomers (pyro attached at the sn-1 or sn-2 position) is produced.
3. Resuspend pyro-lipid isomer mixture in 1 mM $CaCl_2$, 50 mM Tris (pH 8), 0.5% Triton X-100 and 10% methanol at the concentration of 5 mg/ml. Add phospholipase A2 (PLA2) from honey bee venom at 0.1 mg/ml concentration and incubate the solution at 37 °C for 24 hr to digest sn-1 pyro-lipid and to produce the pure sn-2 pyro-lipid isomer.
4. Add additional 2 volumes of chloroform and 1.25 volumes of methanol, extract, and then remove the solvent using rotary evaporation at reduced pressure at 40 °C. Resuspend the cleavage product in 1% MeOH in DCM and purify over a small diol silica column for the isomerically pure pyro-lipid.

2. Preparation of Porphysomes

1. Dissolve pyropheophorbide-lipid, distearoyl-sn-glycero-3-phosphoethanolamine-N-methoxy (polyethylene glycol) (PEG-2000-PE) and cholesterol in chloroform separately, and record the concentrations respectively.
2. Prepare porphysome lipid film in a 12x75 mm borosilicate test tube with a total lipid weight of 5 mg/tube by combining 65 molar % porphyrin-lipid, 5 molar % PEG-2000-PE and 30 molar % cholesterol.
3. Dry the lipid films under a stream of nitrogen gas and let them further dried under vacuum for 1 hr. Store the lipid films at -20 °C under argon until future hydration and extrusion.
4. Add 1 ml phosphate buffered saline (PBS, 150 mM NaCl, 10 mM phosphate, pH 7.4) to each lipid film tube. Freeze the test tube in liquid nitrogen until completely frozen, and then thaw the tube in 65 °C water bath until the sample has all melted. Vortex the tube for a total of 30 sec (3x at 10 sec each, with 5 sec re-heating in 65 °C water bath between each vortexing).
5. Repeat the freeze-thaw for 5 cycles or more upon needed until no large aggregates can be observed in the hydrated solution. Leave the porphysome suspension on ice when done.
6. Assemble the high pressure extruder (10 ml) with two stacked 100 nm polycarbonate filters installed in. Connect the thermobarrel extruder to a thermostatted circulator for 65 °C circulating water bath, and connect the extruder system to a nitrogen tank for high pressure supply.
7. Transfer the porphysome suspension to the extruder chamber using a 9 inch glass transfer pipette. Set the pressure by turning the dial on the nitrogen tank regulator until a reading between 200 and 300 psig is observed as initial extrusion pressure. Open the valve of the pressure regulator gradually until sufficient porphysome flow rate is achieved or a maximum of 800 psig (5,440 kPa) of pressure is reached.
8. After the first extrusion pass is complete, close the pressure control valve and release the pressure of the extruder by opening the pressure relief valve on the extruder top. Refill the extruder with the freshly extruded porphysome solution, and repeat the extrusion for at least 10 times to ensure the final extruded solution is homogeneous.
9. Determine the concentration of final porphysome solution by measuring the absorption of a diluted sample in methanol by UV-Vis spectrometer. Calculate the concentration using the following equation with the extinction coefficient of 97,000 $M^{-1}cm^{-1}$ at 410 nm for pyropheophorbide-lipid.

$$\text{Concentration (nmol/µl)} = \frac{\text{peak absorbance x dilution factor x 1000}}{\text{1cm x extinction coefficient}}$$

10. Measure the final size distribution of porphysomes using a Malvern Zetasizer ZS90 by dynamic light scattering. Dilute porphysome solution in PBS and perform three measurements with 15 runs each for the averaged results.
11. Determine the fluorescence quenching of porphysome by comparing the fluorescence emission of quenched porphysome (1 mM in PBS) and unquenched porphysome (1 mM in 1% Triton X-100) using Spectrofluorometer. Take the spectra before and after the detergent (Tirton X-100) is added, and normalize the signal reading to maximum fluorescence. Set the excitation to be 410 nm, and emission wavelength range to be 600-800 nm.
12. Keep porphysomes solution under argon at 4 °C until future use, and protect it from light by aluminum foil.

3. Preparation of Animal Xenograft Model

1. Culture the KB cells in RPMI-1640 (with 10% FBS), and sustain the cells in 5% CO_2 and moisturized atmosphere at 37 °C.
2. Harvest KB cells using standard cell culture technique. Keep the cell suspension (20,000 cells/ml) in phosphate buffered saline (PBS) on ice for short-term storage until time of injection.
3. Prepare a KB xenografts on nude mice. Nude mice are purchased from Charles River Laboratories when they are 20 g (Category# Crl:NU(NCr)-Foxn1^{nu}, 6-7 weeks-old), given standard diet and bedding (Harlan Laboratories; Diet: Irradiated LM-485, #7912; Bedding: Corncob bedding, ¼", #7097). Sterilize all the animal handling tools (syringe, forceps, and animal pad) prior to experiments. Anesthetize mice with 2% isoflurane v/v in 100% oxygen carrier gas. All animal protocols are approved by the institution (University Health Network in this case) animal care and use committee.
4. Inject tumor cells suspended in PBS subcutaneously to the right flank of mice. Mice are ready to be used after 10 days when tumor reaches diameter of 4-5 mm and thickness of 2-3 mm.

4. *In vivo* Photothermal Therapy

1. Inject porphysomes (containing 750 nmol pyro at 200 µl volume) intravenously via tail vein. Conduct laser irradiation 24 hr post-injection of porphysomes.
2. In this section, conduct all procedures in an enclosed laser safety room. Wear laser safety goggles during laser irradiation.
3. Set up the laser irradiation equipment. Hold the laser fiber and a diffuser by a clamp on a stand. Set the laser power at low level and turn on the laser. Adjust the height of laser fiber to achieve an irradiation area of 9 mm in diameter (DPSS Laser, LaserGlow Technologies, Toronto, Canada), calibrate the laser power to 750 mW (1.18 W/cm^2) using a power meter and then turn off the laser to prepare the animals.
4. Anesthetize the animals with 2% isoflurane v/v in 100% oxygen carrier gas through nose cone no scavenging evident of isoflurane a human health risk hazard). Confirm that the animal is anesthetized deep enough by performing a toe pinch with forceps or fingers to animal's hind paws. The animals are ready for laser irradiation when they are unresponsive to this procedure.
5. Monitor the tumor temperature by an infrared thermal camera (Mikroshot, LUMASENSE Technologies). Place the camera close to the tumor, focus it well and take a pre PTT image as well as a regular white-light image before PTT irradiation starts.
6. Turn on the laser again, and increase the light power to 750 mW. Position the tumor in the center of laser beam to get the tumor fully covered, and start timing the irradiation. Take the tumor temperature image every 5 sec for a one minute laser treatment. Turn off the laser at 1 min. Stop the gas supply of 2% isoflurane, wait until the animal recovers from the anesthesia and put it back to the cage.
7. Keep mice at normothermia animal room under 12:12 hr dark and light cycle according to animal protocols approved by University Health Network. Inject Buprenorphine (0.05 mg/kg, subcutaneously twice a day for a week following the laser irradiation to reduce the pain caused by the scar. Every two days, measure the tumor diameter using a caliper and take a photo. Calculate the tumor volume using the following equation: $V=\pi/6 \cdot a \cdot b^2$, where a is the long diameter, and b is the short one.
8. Sacrifice the animals by putting them in CO_2 chamber when the long diameter of the tumor reaches 10 mm, as it is the defined end point in our laboratory. Perform cervical dislocation following the CO2 treatment. Plot the survival curve.

Representative Results

Pyropheophorbide is conjugated to the phospholipid as a stable lipid monomer (**Figure 1a**) and the conjugates self-assemble to form porphysomes by membrane extrusion method using a high pressure extruder. It is usually difficult to extrude porphysome-lipid suspension during the first 3 cycles of extrusion with relatively slow flow rate. As more extrusions are repeated, the flow rate of extruded solution gradually increases, and pressure can be slightly decreased if needed. Porphysome size, concentration, and quenching efficiency are three important properties to characterize the nanoparticles right after extrusion. Porphysomes with high homogeneity of size distribution (peak around 120 nm) is generated (**Figures 1 b and 1c**) with two main absorbance peaks (410 nm and 677 nm, **Figure 1d**) and can remain stable for over 12 months when being stored at 4 °C. The fluorescence of porphyrin is quenched as high as 99.8% when the nanostructure is intact (**Figure 1e**).

For *in vivo* PTT, heat can be generated rapidly upon the laser irradiation at 24 hr post-porphysome administration (**Figure 2**). Porphysomes with laser irradiation cause the temperature to increase by over 35 °C after 1 min of irradiation, while the laser alone induces a temperature increase of less than 10 °C. It is important that the final tumor temperature reaches 55 °C to ensure complete tumor ablation. Following the PTT irradiation, the tumor usually turns whitish because of the potent thermal effect. The mouse leg becomes a little swollen at the tumor area for around 2 days following PTT. Thermal ablation results in dark brownish eschar on tumors and 100% reduction of tumor volume. Eschars can be observed obviously at 24 hr post-treatment and they gradually recover during the following 2 weeks (**Figure 3a**). Using porphysomes, subcutaneous tumors can be completely eliminated without recurrence (**Figure 3b**), while the tumors in porphysome alone and laser alone control continue to grow (**Figure 3b**), and all reach end point in 2 weeks.

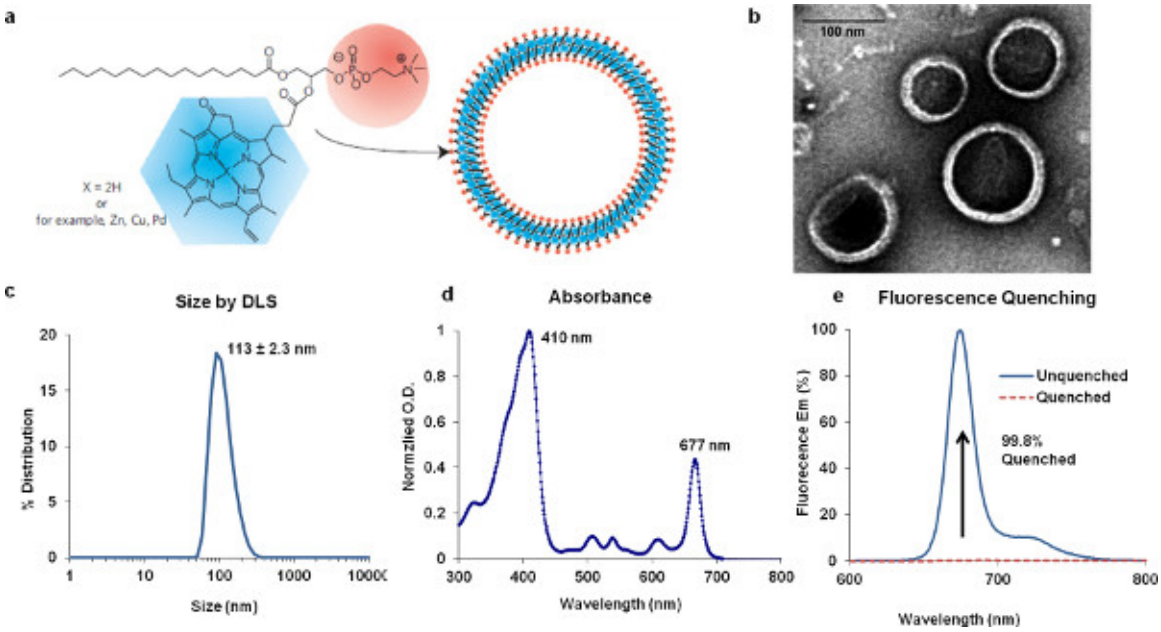

Figure 1. Structure of porphysome nanovesicles and characterizations. a. Schematic representation of a pyropheophorbide-lipid porphysome. **b**. corresponding TEM image. **c**. size distribution of porphysomes after high-pressure extrusion. **d**. Absorbance of the pyropheophorbide-lipid in methanol, normalized to the peak at 410 nm. **e**. fluorescence emission (em) of quenched (red dash line) and unquenched (blue solid line) porphysomes, normalized to maximum fluorescence. Click here to view larger figure.

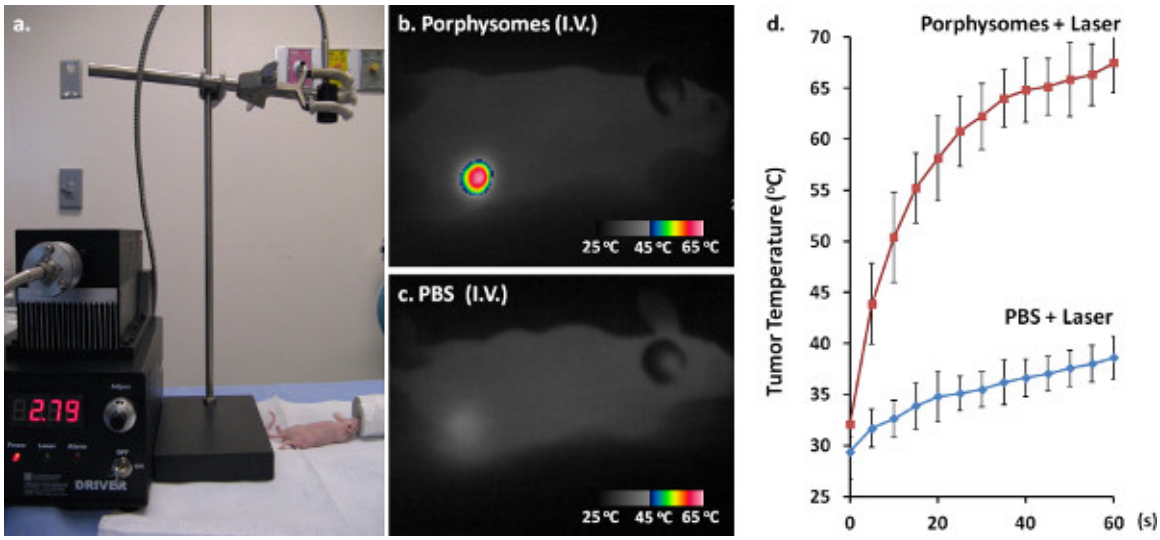

Figure 2. Thermal response during PTT. **a. laser set up for transdermal light irradiation. b** and **c**. Temperature of tumor upon laser irradiation in KB tumor-bearing mice at 24 hr post-injection of porphysomes or PBS. **d**, Maximum tumor temperature during 60 sec laser irradiation (mean +/- SD for 5 mice in each group). Click here to view larger figure.

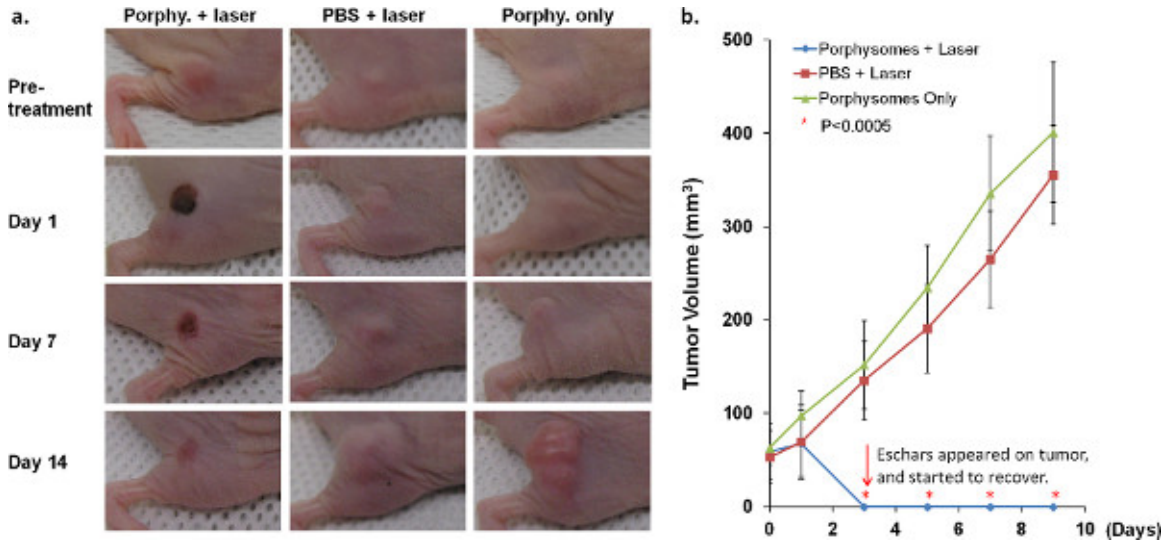

Figure 3. a. Photographs showing therapeutic response to PTT[1], including three groups: porphysomes injection with laser irradiation, PBS injection with laser irradiation, and porphysomes injection alone. b. Average tumor volume after each treatment (n=5, with * represents p<0.0005). Click here to view larger figure.

Discussion

In the development of drug delivery technologies, multifunctional nanoparticles are currently under wide investigations for accurate tracking of the drug delivery vehicle while maintaining a high drug payload. Porphyrin-loaded liposomes have been developed for better pharmacokinetic properties and more efficient delivery than direct administration of porphyrin, but obstacles exist, including the restricted loading amount of porphyrin and rapid redistribution of porphyrin from liposomes to plasma proteins[5,6]. In contrast to conventional liposomal delivery systems, where free porphyrin are inserted into either the core or lipid-bilayer of the liposome, pyropheophorbide (pyro) is now directly and stably conjugated to the phospholipid and self-assemble into liposome-like nanostructures to form porphysomes. As a result, the porphysome bilayer achieves extremely high porphyrin packing density, which leads to structure-dependent self-quenching of pyro fluorescence and high extinction coefficient.

These pyro-lipid conjugates self-assemble into porphysomes in aqueous buffer via similar preparation method of liposomes, such as membrane extrusion method that is commonly used to generate unilamellar vesicles of well-defined size[7-9]. The extrusion starts from a dry lipid film that is hydrated in the aqueous solution and subjected to several freeze-thaw cycles to form multilamellar vesicles with broad size distribution[10]. When the hydrated porphysome lipid film reaches more than 1 mg/ml, manual extrusion becomes physically challenging, so high pressure extrusion is chosen for scaled up production of porphysomes, such as for animal studies when at least 5 mg/ml of porphysome is required[8,9]. To achieve even higher final pyro concentration, porphysome lipid-film can be prepared with higher initial amount. But concentration higher than 10 mg/ml is not recommended, due to the difficulty of extrusion with the 10 ml high pressure extruder. During the extrusion, if the flow rate of the extruded solution coming out is extremely slow (*e.g.* 30 min/ml) even with very high pressure (≤800 psig for safety issue), the filter can be changed to a new set, and the temperature of water-circulation can be increased from 65 °C-75 °C. Size homogeneity of liposomal nanovesicles has been shown to help decrease liver and spleen uptake *in vivo* [11]. Therefore, to obtain a homogeneous and narrow size distribution, the extrusion of porphysome lipid suspension through filter membrane (100 nm) is repeated at least ten times or even more.

We found porphysomes are very stable after extrusion when stored at 4 °C, with negligible change in size or quenching properties for at least one year in our studies (data not shown). It is still recommended to test the porphysome size and quenching efficiency before each animal injection to ensure porphysome intactness.

Porphysomes are the first organic nanoparticles to serve as efficient photothermal enhancers while maintaining the drug delivery capacity and biocompatibility of conventional liposomes. They are based on porphyrins, which have an excellent record for theranostic applications.[12] They have high extinction coefficient comparative to gold nanoparticles (GNPs) for rapid heat generation and efficient thermal ablation[1]. The *in vivo* studies have shown the advantages of porphysome-enabled PTT as an alternative method to conventional cancer treatment. PTT is very simple, with laser treatment relatively localized to the selected irradiation area (9 mm diameter), and very short treatment period (1 min) required to achieve tumor temperature higher than 55 °C for effective thermal ablation. Both the simplicity and high selectivity can help to improve recovery time and reduce the risk of complications for translational therapeutic applications. If other xenograft types that have less tumor accumulation than KB tumor (7.5% ID/g) is used, higher porphysome dose can be IV injected for efficient heat generation upon laser irradiation. During the irradiation process, it is important that laser spot covers the whole tumor area, and tumor temperature is increased to 55 °C or higher for quick and complete thermal ablation at tumors.

Studies now are ongoing to explore the multimodal imaging and therapeutic applications of porphysomes as potent PTT agents in more clinically relevant animal models for future translational study.

Disclosures

We have nothing to disclose.

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