Video Article

Making Conjugation-induced Fluorescent PEGylated Virus-like Particles by Dibromomaleimide-disulfide Chemistry

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Abstract

The recent rise in virus-like particles (VLPs) in biomedical and materials research can be attributed to their ease of biosynthesis, discrete size, genetic programmability, and biodegradability. While they're highly amenable to bioconjugation reactions for adding synthetic ligands onto their surface, the range in bioconjugation methodologies on these aqueous born capsids is relatively limited. To facilitate the direction of functional biomaterials research, non-traditional bioconjugation reactions must be considered. The reaction described in this protocol uses dibromomaleimides to introduce new functionality in the solvent exposed disulfide bonds of a VLP based upon Bacteriophage Qβ. Furthermore, the final product is fluorescent, which has the added benefit of generating a trackable *in vitro* probe using a commercially available filter set.

Video Link

The video component of this article can be found at https://www.jove.com/video/57712/

Introduction

Using nano-sized viral capsids has emerged as an exciting field, which aims to broaden the scope of applications in biomedical research[1,2,3]. Recombinantly expressed virus-like particles (VLPs) are structurally derived from viruses, but they lack the original viral genetic material making them non-infectious proteinaceous nanoparticles. As the surface features are genetically programed and each capsid is expressed identically to the ones before and after it, it is possible to know the location and number of reactive side chains of the amino acids with atomistic precision. In many cases, both the exterior and interior surfaces possess many kinds of solvent exposed amino acid residues, which can feasibly be functionalized through bioconjugation reactions - reactions that form covalent bonds between a biomolecule and a synthetic molecule[4,5].

Bioconjugation reactions help biomolecules of interest have more diverse functionalities in a relatively straightforward fashion. Molecules of interest, such as therapeutic drugs[6], fluorescent tags[7] and polymers[8,9] can be pre-synthesized and characterized before they are attached on the surface of VLPs. A particularly common VLP in biomedical and biomaterials research has been the VLP based upon Bacteriophage Qβ, which, as recombinantly expressed, is a 28 nm icosahedral viral capsid[10]. The most common reaction sites on Qβ are lysines by a wide margin, though we have recently communicated the successful conjugation[11] of dibromomaleimide compounds to the reduced disulfides that line the pores of Qβ via a Haddleton-Baker reaction. The reaction proceeds with good yield and, equally importantly, without losing the thermal stability of the particles. At the same time, this reaction generates conjugation-induced fluorescence, which can be used to track the uptake of these particles into cells. In this work, we demonstrate the conjugation of polyethylene glycol (PEG) onto the surface of Qβ through the Haddleton-Baker reaction, which results in a bright yellow fluorophore. These particles can then be tracked as they are taken in by cells. The protocol herein will help researchers generate new fluorescent PEGylated proteinaceous nanoparticles based upon Qβ, though its principles are applicable to one of the many other VLPs containing solvent exposed disulfides.

Protocol

1. Preparation

1. Make Lysogeny broth (LB) agar and pour plates[12].
2. **Transform BL21(DE3) with a pET28 plasmid containing the wtQβ coat protein sequence.**
    1. Thaw *E. coli* BL21(DE3) competent cells in an ice bath. Place 50 µL of cells in a microcentrifuge tube.
    2. Add 2 µL of plasmid into one tube and gently flick the tube. Then incubate on ice for 30 min.

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3. Heat-shock the cells for 45 s in a water bath that is at exactly 42 °C. Place the tube back in the ice bath immediately after heat-shocking, and incubate for 5 min.
4. Add 950 µL of LB media that does not contain any antibiotic.
5. Shake the culture at 200 rpm for 60 min at 37 °C.
6. Plate 100 µL of the culture on LB agar plates (with Kanamycin) and incubate the plate overnight at 37 °C. Select white colonies when needed.

3. **Make Super Optimal Broth (SOB) media.**
   1. Autoclave two 2 L baffled Erlenmeyer flasks on a superdry cycle.
   2. In an aseptic environment, weigh out and add 20.0 g of tryptone, 5.0 g of yeast extract, 2.469 g of anhydrous magnesium sulfate, 0.584 g of sodium chloride and 0.186 g of potassium chloride to each flask.
   3. Bring the volume to 1 L with ultrapure water in each flask and autoclave on the liquid cycle.
   4. Once the SOB media has reached room temperature after autoclaving, add 1 mL of kanamycin (100 mg/mL) to each liter of media and store at 4 °C.

4. **Make 0.1 M potassium phosphate buffer (pH 7.00).**
   1. Make 1 M potassium monobasic solution by dissolving 68.045 g of potassium monobasic in 500 mL of ultrapure water.
   2. Make 1 M potassium dibasic solution by dissolving 87.09 g of potassium dibasic in 500 mL of ultrapure water.
   3. Add 38.5 mL of potassium monobasic solution and 61.5 mL of potassium dibasic to a 1 L bottle.
   4. Adjust pH to 7.00 if needed, and bring to a volume of 1 L.

5. **Make 5–40% sucrose gradients in 0.1 M potassium phosphate buffer (pH 7.00).**
   1. In 50 mL centrifuge tubes, prepare solutions with 5–40% (increasing in increments of 5%) sucrose dissolved in 0.1 M potassium phosphate buffer (pH 7.00).
   2. Deposit 3.3 mL of 5% sucrose solution at the bottom of a 38 mL round-bottom polycarbonate tube using a long needle syringe and repeat this for five other tubes.
   3. Carefully deposit 3.3 mL of 10% sucrose solution at the bottom of the tube, and carefully remove the needle as to not disturb the gradient. Repeat for the other five tubes.

6. Continue to deposit 3.3 mL layers of sucrose solutions, increasing from 15% to 40% in each tube, while being cautious to not disturb the gradient.
7. When complete, cover the tops of the gradients with foil and store at -80 °C.

2. Expression of Qβ

1. Wipe bench area with 1:1 bleach/ethanol.
2. Make two 3 mL starter cultures in an aseptic environment by adding single colonies of *E. coli* BL21(DE3) into 3 mL of SOB media in an aseptic environment.
3. Grow on a shaker at 250 rpm in a 37 °C and 0% relative humidity (rH) room overnight.
4. **Inoculate starter culture in SOB media:**
   1. Take both 3 mL starter cultures off the shaker and, in an aseptic environment, pour each starter culture into one of two 2 L baffled Erlenmeyer flasks with 1 L of fresh SOB media in each.
   2. Place the inoculated media on a shaker at 250 rpm in a 37 °C and 0% rH room.
5. Grow the bacteria on a shaker at 250 rpm in a 37 °C and 0% rH room until OD_{600} reaches 0.9–1.0.
6. Add 1 mL of 1 M isopropyl β-D-1 thiogalactopyranoside (IPTG) using a P1000 pipette to induce protein expression.
7. Leave the media on the shaker at 250 rpm in a 37 °C and 0% rH room overnight.
8. Remove media from the shaker the following morning and centrifuge it using 1000 mL bottles at 20,621 × g for 1 h at 4 °C to harvest the cells.
9. **Discard the supernatant and collect the cell pellet.**
   1. Pour the supernatant into a flask with about 5 mL of bleach to kill bacteria. This is waste.
   2. Use a spatula to scrape the cell pellet from the bottom of the centrifuge bottle and put the pellet into a 50 mL centrifuge tube.

3. Purification of Qβ

1. Resuspend the cell pellet with ~20–30 mL of 0.1 M potassium phosphate buffer (pH 7.00).
2. Make sure the resuspension has no chunks, and lyse the cells using a microfluidizer processor according to manufacturer's protocol (see **Table of Materials**). Lyse the cells at least twice to increase the yield of the particles.
3. Centrifuge the lysate in 250 mL centrifuge bottles at 20,621 x g for 1 hr at 4 °C.
4. Discard the pellet and measure the volume of supernatant in mL. Multiply that value by 0.265 and add that amount of g of ammonium sulfate to the supernatant.
5. Stir at 4 °C for at least 1 h on a stir plate at 200 rpm to precipitate out the protein.
6. Centrifuge in 250 mL bottles at 20,621 x g for 1 h at 4 °C.
7. Discard supernatant and resuspend the pellet with about 10 mL of 0.1 M potassium phosphate buffer (pH 7.00).
8. Add equal volumes of 1:1 chloroform/n-butanol to the crude sample and mix by vortexing for a few seconds.
9. Centrifuge in 38 mL tubes at 20,621 × g for 30 minutes at 4 °C.

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10. Recover the top aqueous layer using a Pasteur pipette. Be cautious not to take any of the gel like layer that has formed between the aqueous and organic layer.
11. Thaw six 5–40% pre-made sucrose gradients and load about 2 mL of the extract onto each.
12. Ultracentrifuge at 99,582 x g for 16 h at 4 °C with free deceleration.
13. Shine a light emitting diode (LED) light under each tube and a blue band should become visible. Recover these particles with a long needle syringe.
14. Ultrapellet the particles at 370,541 x g for 2.5 h at 4 °C.
15. Discard the supernatant and resuspend the transparent pellet of purified particles with 0.1 M potassium phosphate buffer (pH 7.00).

4. Quantification and Confirmation of the Product

1. Use Bradford Assay to quantify the product[13].
2. Run reducing and non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to confirm the product[14].
   NOTE: Reducing SDS-PAGE is used to confirm the molecular weight of coat protein; non-reducing SDS-PAGE is to confirm the higher order structure.

5. Conjugating DB Compounds on Qβ

1. **Reduce disulfides on Qβ.**
   1. Dissolve 0.0020 g of tris(2-carboxyethyl)phosphine (TCEP) into 1 mL of ultrapure water to make a 100x stock solution.
      NOTE: Prepare fresh TCEP before reduction.
   2. Add 200 µL of Qβ (5 mg/mL) into a microcentrifuge tube.
   3. Follow by adding 20 µL of 100x TCEP stock solution.
   4. Incubate at room temperature for 1 h.

2. **Re-bridge reduced disulfide using dibromomaleimide polyethylene glycol (DB-PEG).**
   1. Dissolve 0.0017 g of dibromomaleimide-polyethylene glycol (DB-PEG) in 100 µL of DMF.
   2. Add 680 µL of 10 mM sodium phosphate solution (pH 5.00).
   3. Add reduced Qβ into the solution of DB-PEG and observe the mixing process under a 365 nm UV lamp. The bright yellow fluorescence can be immediately visualized with a 365 nm handheld UV lamp upon mixing.
   4. Let the reaction proceed overnight at room temperature (RT) on a rotisserie.

3. Purify the reaction mixture by centrifugal filter (COMW = 10 kDa) three times using 1x PBS at 3,283 x g for 20 min at 4 °C.
4. Monitor the conjugation by non-reducing SDS-PAGE and native agarose gel electrophoresis.
   NOTE: VLPs run as intact particles in native agarose gel, and they are separated based on their charge, size and shape.

Representative Results

The dibromomaleimide derivatives can be synthesized through the condensation reaction between dibromomaleimide anhydride and primary amines[15]. Alternatively, a mild synthetic method[16] using N-methoxycarbonyl activated 3,4-dibromomaleimide was exploited here by reacting with methoxypolyethylene glycol (PEG) to yield DB-PEG (**Figure 1**). NMR was used to identify the compound structure (**Figure 2**). Qβ VLP is a 28 nm icosahedral proteinaceous nanoparticle, which is composed of 180 identical coat proteins. The coat proteins tend to form noncovalent interlocking dimers through their α-helical domains with the β-sheets from the adjacent coat proteins[17]. VLPs tend to be selected for their stability at high temperatures, extreme pHs, and in various solvent compositions[18]. In this case, Qβ VLP is more stable than other RNA phages in the *Leviviridac* family owing to 180 inter-strand disulfide bonds located at the five- and six- fold axes of symmetry on the capsid[19] (**Figure 3**). These hexameric and pentameric structures are linked by disulfides, which can be visualized by non-reducing SDS-PAGE[19]. Ten equivalents of TCEP (tris(2-carboxyethyl)phosphine) (0.70 µmol), relative to the disulfides in 1 mg of Qβ (0.070 µmol coat protein, 0.070 µmol disulfides), were used to reduce all the disulfides to generate the reduced Qβ capsids (rQβ) at room temperature in one hour, and non-reducing SDS-PAGE shows that all the higher order structures were reduced to monomeric coat proteins (**Figure 4 and Figure 5A**). The reaction to rebridge them was done as a one-pot synthesis, as the crude rQβ admixture was added directly to 20 equivalents of a solution of DB-PEG (1.4 µmol) in sodium phosphate (10 mM, pH 5.00, 10% DMF) following the one-hour reduction reaction[11]. There was observable bright yellow fluorescence under 365 nm UV lamp (**Figure 5D**) immediately after addition of the DB-PEG. The mixture was then incubated at RT overnight on a rotisserie, followed by purification using centrifugal filter (MWCO = 10 kDa) rinsing with the desired buffer three times to remove excess amounts of small molecules. The Qβ-malemide conjugates were resuspended into 10 mM sodium phosphate solution (pH 5.00) to promote photostability. The conjugation was confirmed by non-reducing SDS-PAGE under UV and coomassie blue staining (**Figure 5A**). All the bands showed fluorescence (**Figure 5A**) under UV which colocalized with the coomassie blue staining, representing a successful conjugation. The integrity of Qβ-PEG conjugates were confirmed by native agarose gel electrophoresis and transmission electron microscopy (TEM) (**Figure 5B, C**).

The fluorescence spectroscopy showed the excitation and emission maxima of Qβ-maleimide (Qβ-M) and Qβ-PEG to be around 400 nm and 540–550 nm, respectively (**Figure 6**). This aligns with the commercially available GFP-uv filter set, whose excitation wavelength is 405 nm and emission wavelength is 500–540 nm. The convenient alignment of the photophysical properties of the conjugates with the commercially available filter sets permit using Qβ-PEG as an *in vitro* probe, which was done and imaged in **Figure 7**. Qβ-PEG (200 nM) was incubated with Mouse Raw-264.7 cells in serum-free DMEM medium, followed by nucleus staining. Colocalization images in **Figure 7** shows that yellow fluorescent particles were uptaken by Raw-264.7 cells and can be tracked after four hours of incubation. The unfunctionalized Qβ VLPs show negligible fluorescence.

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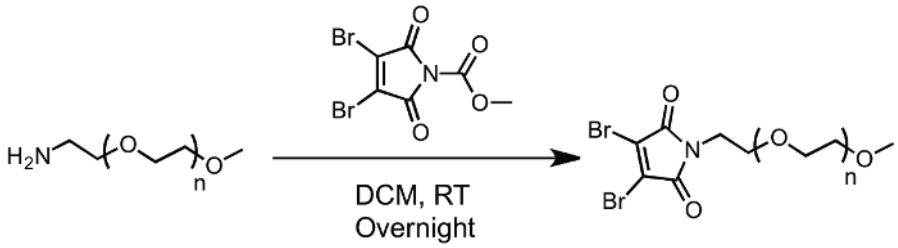

Figure 1: Synthetic scheme of DB-PEG. Please click here to view a larger version of this figure.

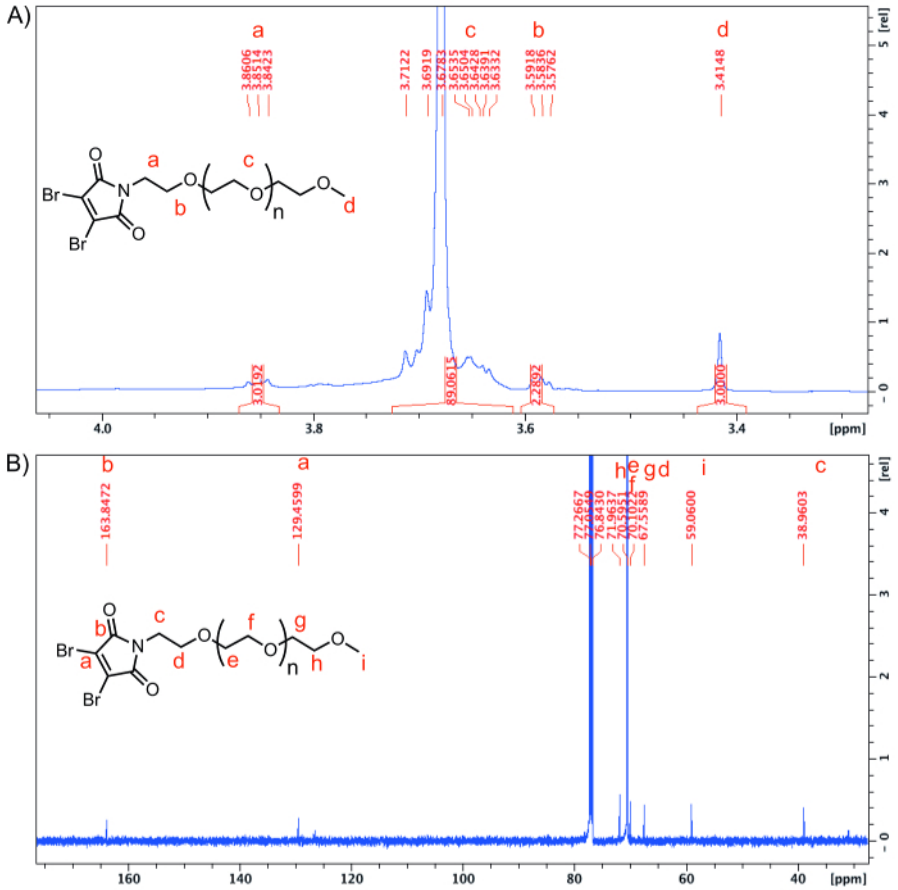

Figure 2: NMR characterization. (A) ^{1}H NMR and **(B)** ^{13}C of DB-PEG. Please click here to view a larger version of this figure.

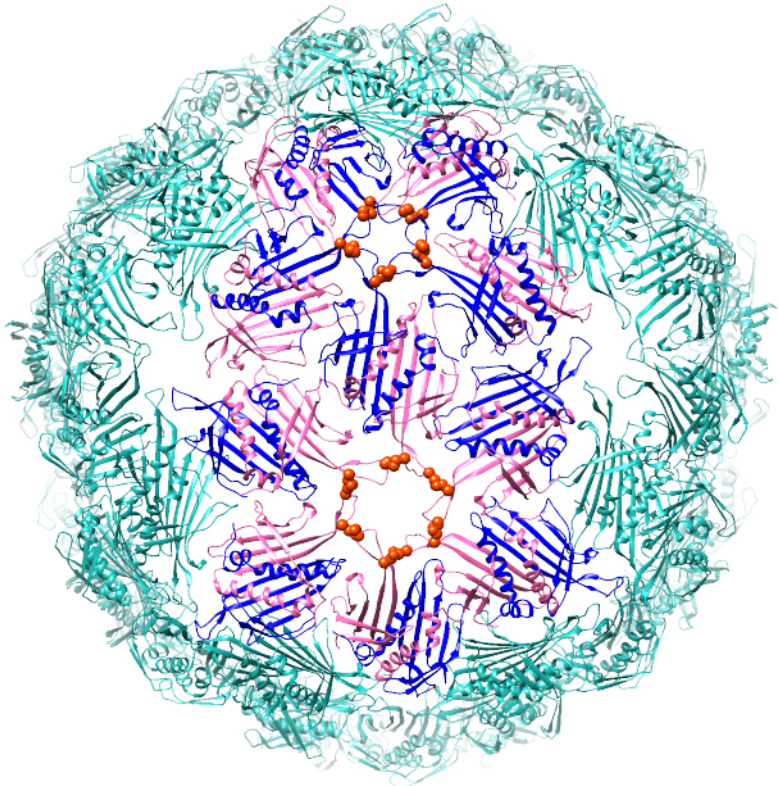

Figure 3: Crystallographic structure of Qβ VLP capsid as processed in Chimera (PDB ID: 1QBE). Two cysteine residues (Cys 74 and Cys 80) are shown in orange.

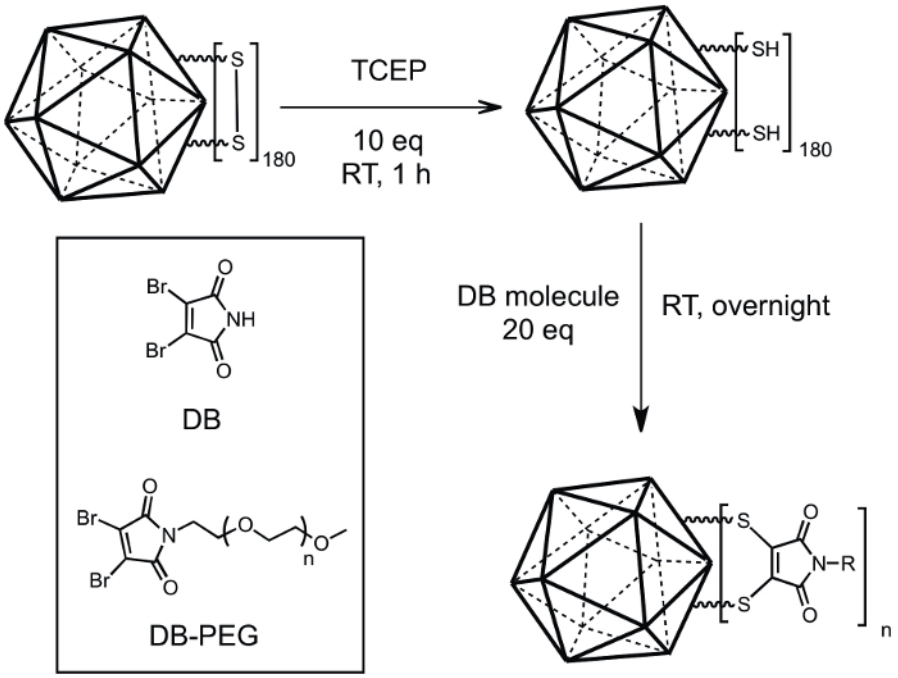

Figure 4: Conjugation scheme of Qβ-maleimide (Qβ-M) conjugates. Qβ (0.07 µmol of disulfides) was reduced using 10 equivalents of TCEP (0.7 µmol) at RT for one hour followed by addition of 20 equivalents of dibromomaleimide compounds (DB and DB-PEG) (1.4 µmol). Please click here to view a larger version of this figure.

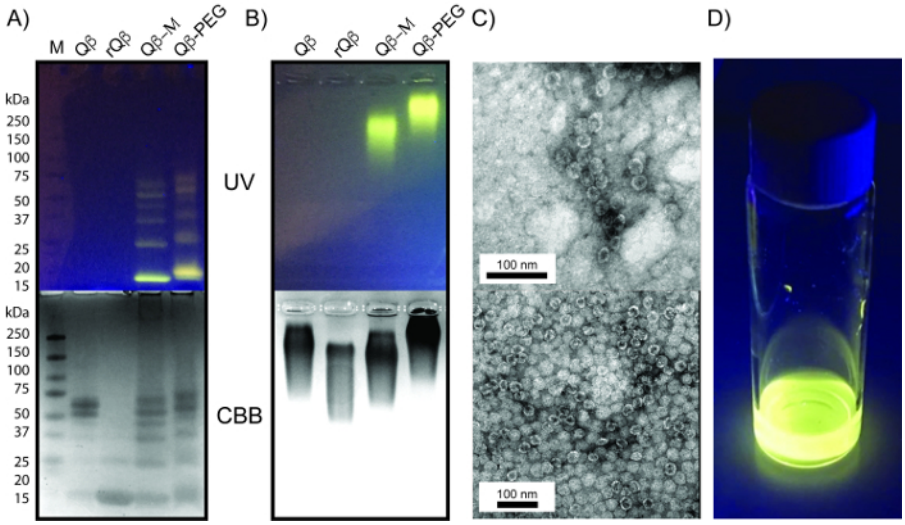

Figure 5: Characterization of Qβ, rQβ, Qβ-M and Qβ-PEG. (A) Non-reducing SDS-PAGE and **(B)** native agarose gel of Qβ, rQβ, Qβ-M and Qβ-PEG under UV (top) and coomassie blue staining (bottom). **(C)** TEM micrograph of Qβ-M (top) and Qβ-PEG (bottom). **(D)** Photograph of Qβ-PEG reaction mixture under 365 nm UV illumination. Please click here to view a larger version of this figure.

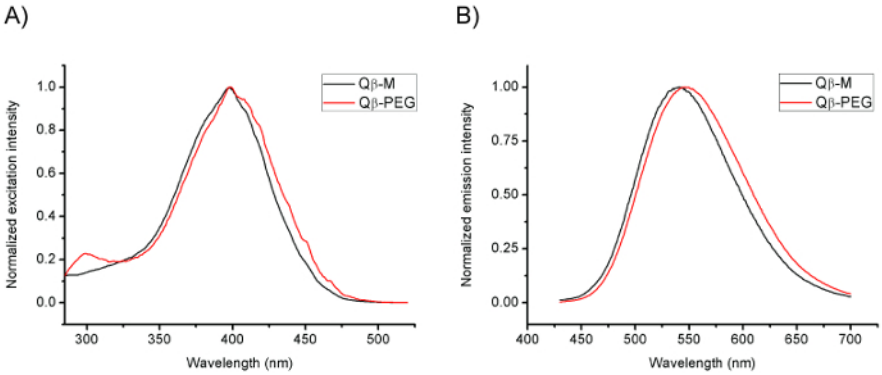

Figure 6: Fluorescence spectra. Fluorescence excitation **(A)** and emission **(B)** spectra of Qβ-M and Qβ-PEG in 0.1 M of potassium phosphate buffer (pH 7.00). The excitation maximum is around 400 nm and emission maximum is around 540–550 nm. Please click here to view a larger version of this figure.

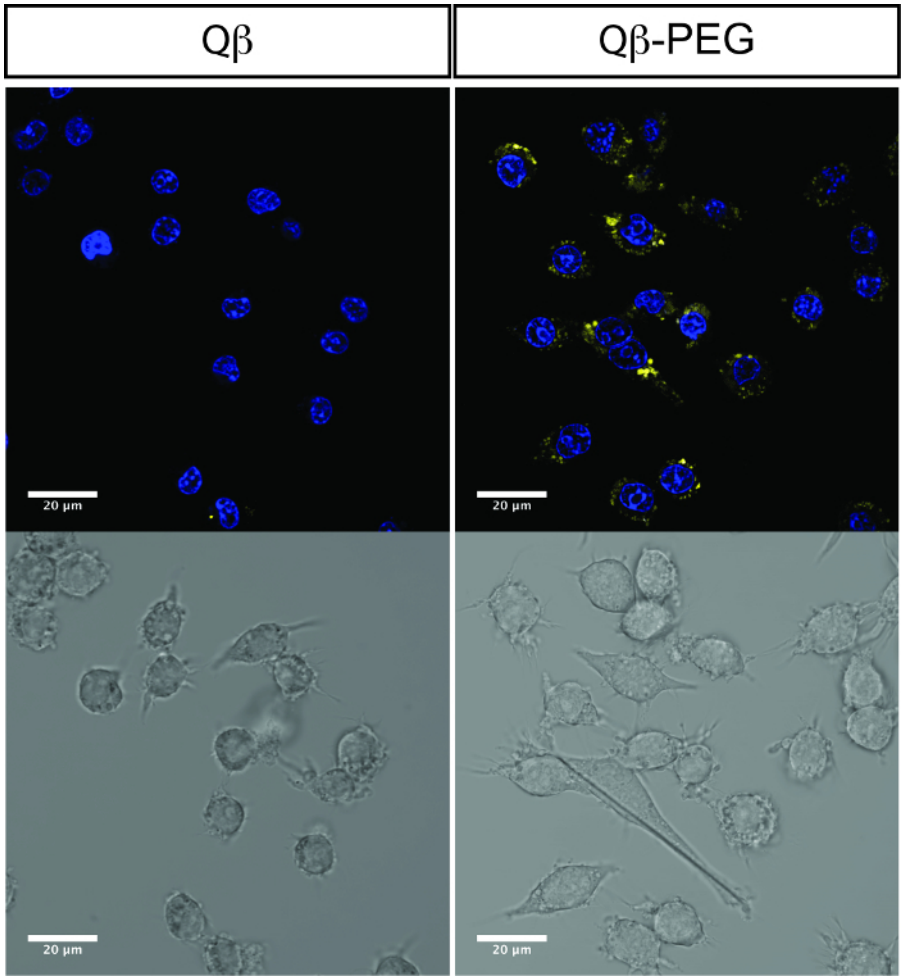

Figure 7: Confocal fluorescence images of Qβ-PEG conjugate in macrophage 264.7 cells. Blue: NucRed Live 647 ReadyProbes Reagents. Yellow: Qβ-PEG. Top images: merged blue and yellow channels. Bottom images: bright field images. Filter sets: uv-GFP (λ_{ex} = 405 nm, λ_{em} = 500–540 nm), Cy5. Time of incubation was 4 h. Please click here to view a larger version of this figure.

Discussion

Compared to smaller protein purification, a unique step in purifying bacteriophage Qβ is the sucrose gradient centrifugation. After the chloroform/n-butanol extraction step, Qβ is further purified using 5-40% sucrose gradients. During centrifugation, particles are separated based on their sizes. Larger particles travel to the higher density region, while smaller particles stay in the lower density region. Qβ travels to the lower third of the gradient and remains there while smaller protein impurities are trapped at the top of the centrifuge tube. The colloidal suspension of Qβ shows strong Tyndall scattering in the sucrose gradient, which can be seen as a blue band when an LED light is shined from underneath. This band is then easy to extract using a long syringe needle. Qβ in sucrose is then pelleted by ultracentrifugation, yielding a clear pellet. The pellet can be resuspended into the desired buffer or further purified by fast protein liquid chromatography (FPLC). The purity of the resulting particle can be confirmed by SDS-PAGE.

TCEP is not stable in phosphate buffer, so a 100x TCEP stock solution should be freshly prepared in water right before the reduction. In addition, TCEP neither contains free thiol nor reacts towards other amino acids, so it is not necessary to remove the excess of TCEP prior to performing the conjugation reaction. Dibromomaleimide compounds are dissolved in pH 5.00 sodium phosphate solution, followed by adding the reduced Qβ. At pH 5.00, cysteine (reduced disulfide) is protonated, which promotes the conjugation reaction with dibromomaleimide by preventing re-oxidation back to a disulfide. Under 365 nm UV illumination, the reaction mixture emits yellow fluorescence immediately after reduced Qβ was added into DB compounds. Differently from attaching pre-synthesized fluorophores on Qβ, this fluorescence is induced by the conjugation reaction. The fluorophore is formed as soon as the new bonds between sulfur and the maleimide ring are created. Fluorescence spectra shows that the excitation (400 nm) and emission (550 nm) maxima fit the uv-GFP filter set in a confocal microscope (λ_{ex} = 405 nm, λ_{em} = 500−540 nm). Qβ-PEG was then incubated with macrophage Raw 264.7 in serum-free DMEM. After four hours of incubation, Qβ-PEG was uptaken and punctate yellow fluorescent compartments can be visualized inside the cells. One fact that needs to be mentioned is the fluorescence may be transferred for some extent to bovine serum albumin (BSA) in serum or other cysteine-rich substances inside the lysosomes or the late endosomes of cells. Over time, this will weaken the fluorescence inside the cell for cell tracking applications; however, it also provides an opportunity for a new design for drug delivery systems.

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In conclusion, we have demonstrated conjugating DB-PEG on Qβ VLP through dibromomaleimide-thiol chemistry. The all-in-one conjugation reaction not only functionalizes the disulfides along the pores on VLP with PEG, but also makes it a fluorescently labeled proteinaceous nanoparticle.

Disclosures

The authors declare that they have no competing financial interests.

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