Self-Assembly of drug-loaded liposomes on genetically engineered protein nanotubes: A potential anti-cancer drug delivery vector **

Supporting Information

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Experimental Section

***Genetic engineering, purification and amplification of Salmonella flagella*:** The following two pairs of synthetic oligonucleotides (forward and reverse primers) were designed to encode E_8 and synthesized by Invitrogen:

5' –GA TCT cga ggt gat gaa gag gaa gag gaa gag gaa gaa C-3'

5' –TC GAG ttc ttc ctc ttc ctc ttc ctc ttc atc acc tcg A-3'.

By annealing, the forward and reverse primers were combined into a double stranded DNA. At the same time, two restriction enzyme sites of *Xho*I at N-terminal and *Bgl*II at C-terminal were formed for oriented cloning. The annealed primers with two restriction enzyme sites at two ends were inserted into the linearized plasmid PLS411 by *Xho*I and *Bgl*II. Enzymes. Then the plasmids were transformed into a flagellin deficient salmonella strain of SL5928. Individual colonies were picked on RMG-Amp (0.1 %) plates and the recombined plasmid was confirmed by DNA sequencing. In order to increase the motility and length of engineered flagella, the SL5928 was inoculated into semisolid medium. Finally, from the advancing edge of the growth on the semisolid medium, the highly motile salmonella were inoculated in 5 mL of LB-Amp (0.1 %) media at 37 °C with shaking (250 rpm) overnight. The cloudy cultures were then transferred to 1 L of LB-Amp (0.1 %) media and grown at 37 °C with shaking (250 rpm) until the OD reached about 0.5. The culture was centrifuged at 7000 *g* for 15 min at 4 °C. The cell pellets were resuspended into 50 mL of PBS buffer (pH = 7.4) and centrifuged again at 7000 g for 15 min. This step was repeated twice to remove the impurity. Finally, the cell pellet was resuspended in DI water and vortexed on a vortex mixer for 3 minutes. Due to the vortexing, the flagella were detached from the cells and suspended in the solution. The flagella were collected by centrifugation at 10000 *g* for 15 min. The supernatant containing flagella was frozen at -20℃ for further usage. A sample (10 μl) of appropriate flagella dilution was taken on TEM copper grid and examined under transmission electron microscope. The concentration of flagella solution was determined by using the BioRad assay kit with Coomasie blue as internal standard.

Preparation of ZnPc-loaded Liposomes. ZnPc was loaded in the cationic liposomes, by using the detergent depletion method, with cetyltrimethylammonium bromide (CTAB) as the depleting surfactant.[1, 2] The cationic liposome consisted of 1,2-dioleoyl- 3-trimethylammonium propane chloride (DOTAP) and 1,2 Dioleoyl-sn-glycero-phosphocholine (DOPC) in a mass ratio of 30/70. A stock solution of ZnPc was dissolved in 1-methyl-2-pyrrolidone at 50 °C, and added to the chloroform mixture of DOTAP/DOPC such that the ratio of ZnPc:lipid was kept constant at 1: 100. The ZnPc-lipid solution was dried with a stream of nitrogen gas and a transparent film was deposited in the bottom of a glass vessel. To the dried film was added a highly concentrated solution of CTAB (3:1 detergent/lipid molar ratio), prepared with a phosphate buffer (10 mM NaH_2PO_4, pH 7.0, 100 mM NaCl). Samples were then sonicated until clarity, to form mixed micelles, and extensive dialysis was performed using a dialysis bag (MWCO 6-8000) with 3 changes of phosphate buffer solution. After dialysis residual CTAB was removed from the liposome mixture by eluting the suspension through a sephadex G-50 column with the same solvent. The resultant liposome solution was filtered by extrusion through a polycarbonate membrane with 200 nm pores (Avestin, Ottawa, Canada) to remove ZnPc that was not incorporated in the lipid bilayer of the liposomes.

Characterization of liposome and flagella-liposome complex. The liposome solution and flagella-liposome complexes were characterized by using the transmission electron microscopy (TEM), dynamic light scattering (DLS) and zeta potential measurements. TEM images were recorded on a Zeiss 10 microscope, with copper grids used as the sample holders. Liposomes, flagella-liposome complexes were stained with a solution consisting of 5% phosphotungstic and 3% trehalose, pH 7. A drop of liposome suspension was first applied to the copper grid, allowed to adsorb on the grid for 2 min, and then blotted with filter paper. To the wet grid was added a drop of the stain and immediately blotted. For the flagella, the sample was dried before the stain was added to the grid, whereas for the flagella-liposome complexes, the sample was fixed with 1% glutaraldehyde for 5 min before staining. Zeta potential and DLS measurements were performed with a ZetaPals, a zeta potential analyzer from Brookhaven instruments Corporation. Zeta potentials were automatically calculated from the electrophoretic mobility by means of the Hemholtz-Smoluchowski relation. The stock solutions were diluted at least 10 times to meet up with instrumental limitations.

Fluorescence Studies. The spectroscopic methods were used to characterize ZnPc loaded in liposome formulation. UV-vis absorption spectra were measured by using a Shimadzu UV-2101 PC UV-Vis scanning spectrophotometer. The concentration of ZnPc in the liposome was estimated by monitoring the absorbance at 673 nm (Q-band, molar absorption coefficient

ε = 2.41 x 10^4 M^{-1} cm^{-1}). The concentration of ZnPc in all liposome formulations was fixed at 2 x 10^{-6} M and was estimated by using the Beer-Lambert Law (A = εlc). Steady-state fluorescence measurements were carried out by using a Shimadzu RF-5301 PC spectrofluorophotometer. The excitation wavelength was fixed at 670 nm, whereas the emission was collected in the 600-800 nm range. The spectra were collected in 3 nm slit width for both the excitation and emission monochromators, respectively. The temperature of the cuvette holder was maintained at 25 ± 2 °C by using a water-circulation bath. The comparative method was used to calculate the fluorescence quantum yield (Φ_F) of ZnPc in liposome according to the following equation and by using ZnPc in DMSO as the standard (Φ_F = 0.18)[3]

$$\phi_F = \phi_{F(std)} \frac{F.A_{std}.\eta^2}{F_{std}.A.\eta_{std}^2}$$

where F and F_{Std} are the areas under the fluorescence curve of the sample and the standard, respectively. Similarly, A and A_{Std} are the absorbance of the compound and the standard at the excitation wavelength, η and η_{Std} are the refractive indices of solvents used for the sample and the standard, respectively. Fluorescence quenching with methyl viologen ($MV^{2+)}$ were carried out by measuring the fluorescence intensity after sequentially adding small amounts of MV^{2+} to aliquot of the ZnPc loaded flagella-liposome complex. The emission intensity was monitored after excitation at 600 nm and the maximum of the emission spectrum at 604 nm was used to analyze the quenching data by using the Stern-Volmer equation below.[4, 5]

$$\mathbf{I_0/I=1+K_{SV}[Q]}$$

Where I_0 and I are the fluorescence intensities in the absence and presence of the quencher, respectively, K_{SV} is the Stern-Volmer quenching constant, and [Q] is the concentration of the quencher (MV^{2+}) in the sample.

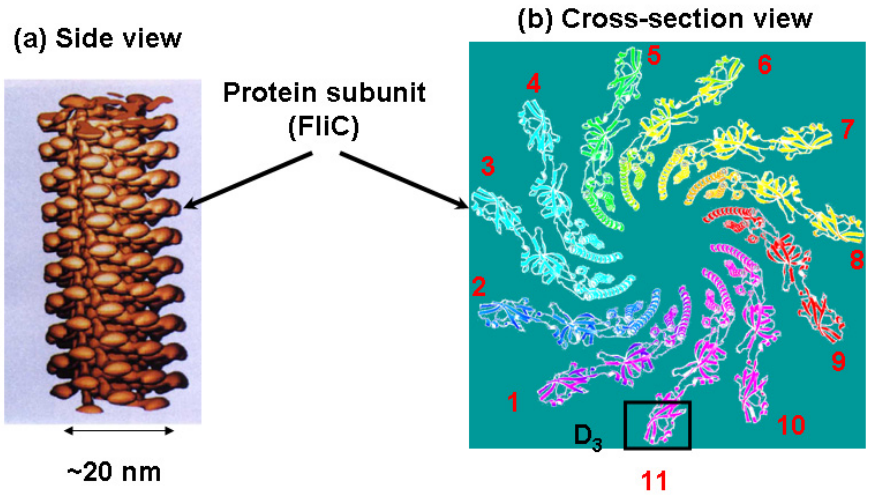

Figure S1 (a) A side-view[6] of a section of a bacterial flagellar filament that protrudes on the bacterial cell surface and is made of several thousand copies of protein subunit called flagellin or FliC. (b) top-view[7] of the flagellar filament showing the organization of flagellin (FliC) viewed along the long axis of the flagellar filament. There are 11 FliC proteins per 360^{o} turn. The black square on No. 11 FliC shows the D3 domain where foreign peptides (e.g., a negatively charged peptide E_8 in this work) can be inserted (i.e., displayed) for each FliC without affecting the polymerization of FliC into a filament. Each FliC can be pictured as a finger and the foreign peptide is inserted in the finger tip region of the D3 domain.[8] The variable D3 domain is framed in black in (b), and is where the foreign peptide can be genetically inserted (i.e., displayed).

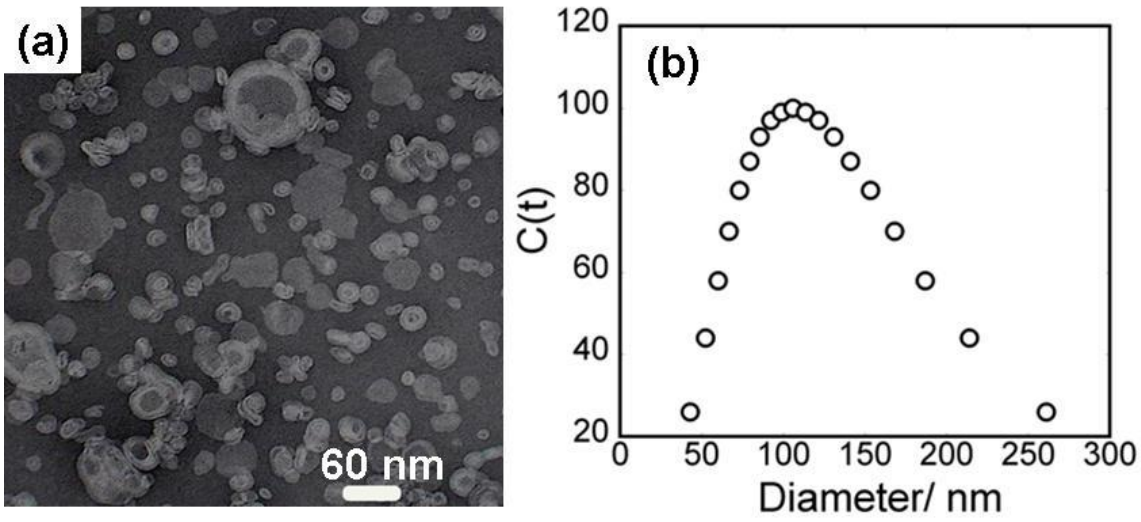

Figure S2. (a) TEM microscope image and (b) the size distribution profile for ZnPc loaded liposome. In (a), the liposomes show different sizes because some liposomes fuse to form larger ones during TEM sample preparation.

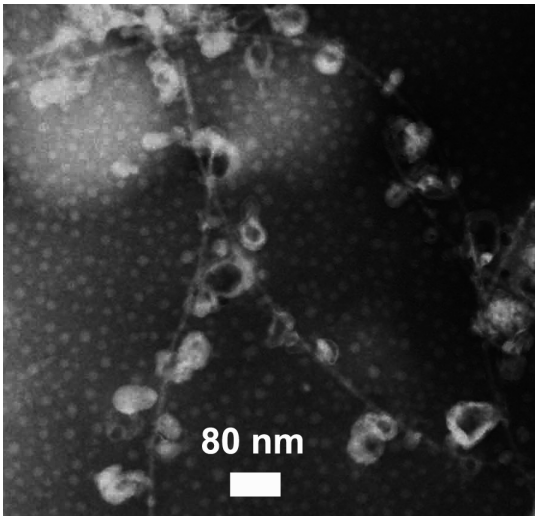

Fig. S3. TEM image of ZnPc-loaded liposomes immobilized on flagella (shown in Fig. 2b) after being incubated in plasma at 37 ^{o}C for 90 min. (Note: the smaller particles around the flagella-liposome complexes are not liposomes which are much larger. They were from the home-made supporting Formvar film on the TEM grid because they were also found on the blank film before the deposition of flagella-liposome complex).

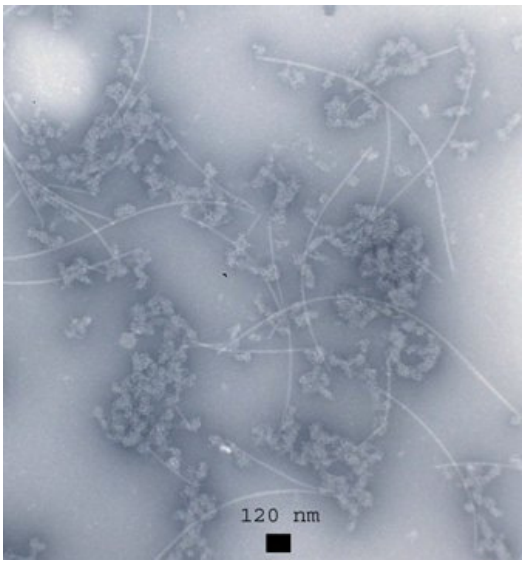

Figure S4. TEM image of ZnPc loaded liposome 30 min after addition of wild type flagella.

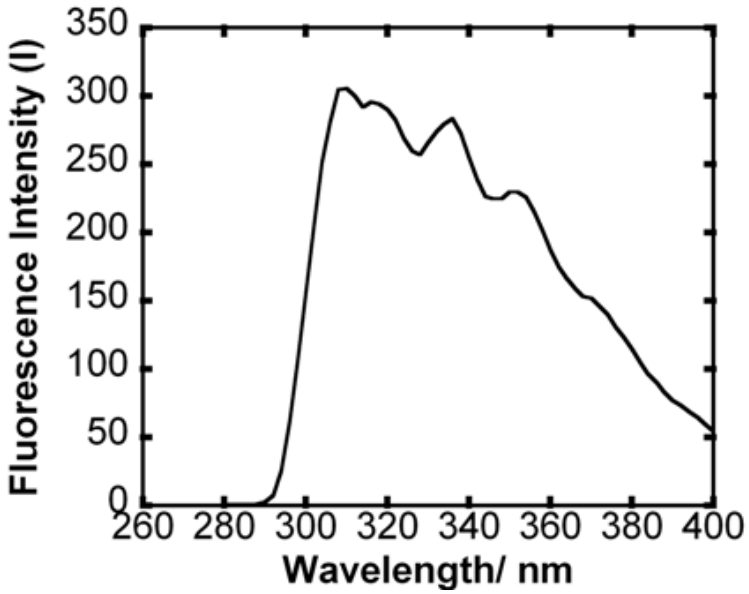

Figure S5. Fluorescence spectrum of genetically engineered flagella that display Glu_8 on the surface in buffer solution pH 7.

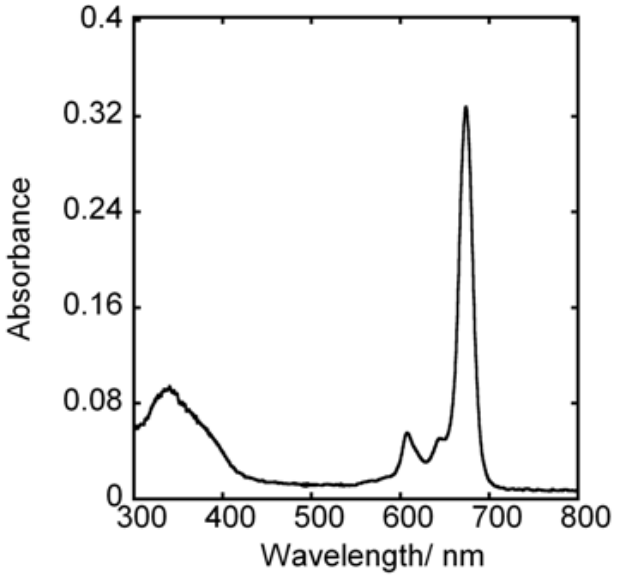

Figure S6. Absorption spectra of 2 x 10^{-8} M ZnPc in the flagella-liposome complex, at a flagella concentration of 0.42 x 10^{-3} mg/mL.

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