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Dual Stimuli-Responsive Vesicular Nanospheres Fabricated by Lipopolymer Hybrids for the Tumor- Targeted Photodynamic Therapy

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ABSTRACT

Smart delivery system of photosensitizer chlorin e6 (Ce6) has been developed for targeted photodynamic therapy (PDT). Simple self-assemblies of the mixtures comprised of soybean lecithin derived phosphatidylcholine (PC), phosphatidylethanolamine–poly(L-Histidine)₄₀ (PE–p(His)₄₀) and folic acid (FA) conjugated phosphatidylethanolamine-poly(*N*-isopropyl acrylamide)₄₀ (PE–p(NIPAM)₄₀–FA) in different ratios yield smart nanospheres characterized by i) stable and uniform particle size (~ 100 nm), ii) positive surface charge, iii) high hydrophobic drug (Ce6) loading efficiency up to 45%, iv) covalently linked targeting moiety, v) low cytotoxicity, and vi) smartness showing p(His) block oriented pH and p(NIPAM) oriented temperature responsiveness. The Ce6-encapsulated vesicular nanospheres (Ce6@VNS) were used to confirm the efficiency of cellular uptake, intracellular distribution, and phototoxicity against KB tumor cells comparing to free Ce6 at different temperature and pH conditions. The Ce6@VNS system showed significant photodynamic therapeutic efficiency on KB cells than free Ce6. A receptor-mediated inhibition study proved the site-specific delivery of Ce6 in targeted tumor cells.

KEYWORDS: Cancer therapy, Drug delivery, Lipopolymer, Photodynamic therapy, Stimuli responsive, Vesicle

INTRODUCTION

To date, various cancerous tumors have been treated by conventional anticancer therapies such as chemotherapy and photodynamic therapy (PDT).¹ The PDT using photosensitizers (PSs) has received more attention as a versatile therapeutic cancer treatment.² Specific wavelengths of light activate the photosensitizer to generate highly reactive singlet oxygen species which non-invasively treat diseased tissues, while causing minimal nonspecific damage to normal tissues. Additionally, the PDT offers easy detection and tracking of tumors both *in vitro* and *in vivo*; fluorescence emission from the photosensitizers aids the imaging and quantification of photosensitizers during the treatment.³ Even though the PDT-based clinical trials are promising, a few problems such as the weak absorption of PSs in the red region of visible light and their poor water solubility need to be overcome. Moreover, the majority of PSs have an absorption band below 650 nm.⁴ Among PDT candidates, chlorin e6 (Ce6) possesses advantages such as a strong absorption band at 663 nm, low cytotoxicity, and almost no side effects to skin. It penetrates deeply into tissue and produces higher reactive oxygen species (ROS).⁵

The selective accumulation of the PSs in tumors strongly dependent on the interaction between the hydrophobic macrocycle and low-density lipoprotein (LDL) in the blood due to the highly expressed LDL receptors in the tumor cells.⁶ The lipid formulation of PSs has been attempted to circumvent the drawbacks of reticuloendothelial system (RES) trapping and renal clearance of PSs-LDL complex.^{7,8} Most of the liposomal formulations showed lack of selectivity and drug leakage during cancer treatment either by chemotherapy or PDT. The leakage of the drug by Fickian diffusion could not avoid by the incorporation of polyethylene glycol (PEG) into nanocarriers (NCs) as a longevity moiety.⁹ Additionally, PEG does not provide any selectivity of drug carriers in delivering therapeutics into the cancer cells. Recently, our group reported a

poly(L-histidine) (p(His)) incorporated lipopolymer for the controlled delivery of doxorubicin (Dox).¹⁰ The resulting self-assembled NCs showed a reasonable circulation stability (72 h) and a fusogenicity, where the pH sensitive p(His) block disrupted the membranes of acidic subcellular endosomes because it enhances the endosomal escape.¹⁰ In this aspect, p(His)-based hybrid materials have great developmental potential as pH responsive liposomal NCs in therapeutic delivery systems.¹¹

The multi-responsive delivery vehicles responding pH, temperature, redox, and/or magnet have recently emerged as a promising platform for tuning the “smartness” of nanocarriers.^{12,13} Specifically pH/temperature dual responsive NCs have been widely studied because they exploit the intrinsic differences in the relative acidity and temperature between solid tumors and the surrounding normal tissues.¹⁴ Poly(*N*-isopropylacrylamide) (p(NIPAM)) has been widely adopted as a temperature responsive moiety since its lower critical solution temperature (LCST) is close to the physiological temperature of the human body.^{15,16} Accordingly extensive study has been made for the development of stimuli responsive polymeric drug carriers for the controlled delivery of anticancer agents;¹² however, little effort has gone towards developing stimuli responsive NCs for the controlled delivery of PSs for PDT.¹⁷

Along with the stimuli responsive NCs, targeting species decorated NCs enhance the endocytosis in the controlled delivery of the therapeutics with respect to malignancies. In particular, the well-known tumor-associated folate receptor is a good target and is overexpressed in many tumors of the breast, lung, kidney, and brain due to the high affinity for folate in the receptor of cancer cells.¹⁸ Reports have also addressed the importance of cationic liposomes that have high affinity for angiogenic endothelial and tumor cells.¹⁹ The cationic liposomes significantly enhanced the endocytic uptake of PS into cells in the presence of serum.²⁰ In

addition nanoparticles decorated by the zwitterionic phosphoryl choline also showed enhanced cellular internalizations than unmodified counterparts.²¹ This high affinity allows cationic/choline enriched liposomes to deliver the drug into tumor cells, effectively inducing damage to cancer cells.

The goal of this study was is to develop a new lipopolymer PDT formulation to circumvent the drawbacks of the conventional liposomal formulations showing low retention and low specificity of NCs. For this dual-responsive lipopolymer hybrid systems bearing phosphatidylcholine (PC), pH responsive phosphatidylethanolamine (PE)-p(His) conjugate, and temperature responsive FA conjugated PE-p(NIPAM) conjugate have been developed. The resulting Ce6-encapsulated vesicular nanospheres (Ce6@VNS) are intensively tested to confirm the efficiency of cellular uptake, intracellular distribution, and phototoxicity against KB tumor cells comparing to free Ce6 at different temperature and pH conditions.

EXPERIMENTAL SECTION

Materials. 1-Dodecanethiol (98.5%), tetramethylammonium bromide (98.5%), carbon disulfide (99.9%), triethylamine (TEA, 99.5%), 4-(dimethylamino)pyridine (DMAP, 99%), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 99.0%), FA (97%), 2,2'-azobis(2-methylpropionitrile) (AIBN 98%), propargyl alcohol (98%), 5-bromovalerate (97%), bromotris(triphenylphosphine)copper(I) ($\text{Cu}(\text{PPh}_3)_3\text{Br}$), Leupeptin, 2',7'-dichlorofluorescein diacetate (DCFH-DA), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and propidium iodide (PI) were purchased from Sigma-Aldrich and used without further purification. *N*-Isopropylacrylamide (NIPAM, 97% Acros Organics) was recrystallized from hexane and solvents such as *N,N*-dimethylformamide (DMF), 1,4-dioxane, acetone and chloroform were purified according to standard procedures before use. Ce6 was obtained from Frontier Scientific,

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3 Inc. (Logan, USA) and *N*-tert-butoxycarbonyl-*N*-benzyl-L-histidine (Boc-L-His-(Bzl)-OH) was
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5 purchased from Bachem. Fluorescein isothiocyanate (FITC)-Annexin V was obtained from Santa
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7 Cruz Biotechnology, Inc. (Santa Cruz, USA). Cell culture materials were purchased from
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9 Invitrogen (Carlsbad, USA). PC and PE were simultaneously extracted from soybean lecithin
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11 (Solae Co., South Korea) by using a mixed solvent consisting of ethanol and chloroform
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13 according to the reported procedure.²²
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18 **Instrumentation and measurements.** ¹H NMR (400 MHz), ¹³C NMR (100 MHz), and
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20 ³¹P NMR (161.9 MHz) spectra were recorded on a Varian INOVA 400 NMR spectrometer. The
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22 temperature-dependent ¹H NMR measurements were performed after the sample tube was
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24 allowed to equilibrate at each preset temperature for approximately 10 min. Chemical shifts are
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26 presented in parts per million (ppm) relative to the residual solvent peaks as the internal standard.
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28 Peak multiplicities in ¹H NMR spectra are abbreviated as s (singlet), d (doublet), t (triplet), m
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30 (multiplet), and br (broad). Column chromatography was performed using a Combi-Flash
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32 Companion purification systems (Teledyne ISCO) using 300–400 mesh silica gel. UV-vis
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34 turbidimetry experiments were carried out for LCST determination on a Shimadzu UV-1650 PC
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36 equipped with a temperature controller. Fourier transform infrared (FT-IR) spectra were recorded
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38 on a Shimadzu IR prestige 21 spectrometer. The spectra were taken on KBr discs in the range
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40 4000–600 cm⁻¹. The molecular weight (MW) and polydispersity index (Đ) of the polymers were
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42 measured on a Waters GPC system, which was equipped with a Waters 1515 HPLC solvent
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44 pump, a Waters 2414 refractive index detector, and three Waters Styragel High Resolution
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46 columns (HR4, HR2, HR1, with effective MW ranges of 5000–500,000, 500–20,000, and
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48 100–5000 g/mol, respectively) at 40 °C using high-performance liquid chromatography (HPLC)-
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grade tetrahydrofuran (THF) containing 0.1 N LiBr or HPLC-grade DMF as eluent at a flow rate of 1.0 mL/min. Monodisperse polystyrenes were used to generate the calibration curve.

Dynamic light scattering (DLS) measurements were carried out using a Nano ZS90 zeta potential analyzer (Malvern Instruments, Ltd., U.K.) with a He–Ne laser (633 nm), 90° collecting optics, and a thermoelectric Peltier temperature controller. Block copolymer solutions (2 mg/mL) were filtered through a 0.5-μm filter prior to use. Particle morphology was analyzed by transmission electron microscopy (TEM). TEM was performed using a JEOL–1299EX electron microscope with an accelerating voltage of 80 keV. TEM samples were prepared in grids with formvar film and treated with oxygen plasma (from a Harrick plasma cleaner/sterilizer) for 15 s to make their surface hydrophilic. A TEM grid was then floated on top of the bead with the hydrophilic face in contact with the solution; the aqueous solution was blotted away with a strip of filter paper and the samples were dried overnight at room temperature.

Synthesis of benzyl-*N*-carboxy-*L*-histidine anhydride (Bn-His-NCA). Boc-*L*-His(Bn)-OH (2.5g) was suspended in anhydrous 1,4-dioxane (10 mL), to which a solution of PCl₅ (1.8 g) in 1,4-dioxane (20 mL), was added to form Bn-His-NCA at 25 °C under stirring. Within a few minutes, a clear solution was obtained and filtered through a glass filter. Crystals of Bn-His-NCA were obtained after the addition of the filtrate to an excess of diethyl ether. The product was subsequently washed and dried under vacuum at 50 °C. ¹H NMR (400 MHz, DMSO d₆) δ = 2.78 (d, 2H, HC-CH₂-C), 4.83 (t, 1H, N-CH-CH₂-), 5.15 (s, 2H, N-CH₂-C), 6.5–7.4 (m, 2H, N=CH-N-, N-CH=C-), and 7.15–7.4 (m, 5H, Ar-H) ppm.

Synthesis of PE-p(His)₄₀ conjugate. To a flame-dried nitrogen-purged schlenk tube, 0.69 g (0.1 mmol) PE-NH₂ was added under nitrogen, followed by the addition of 5 mL

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3 anhydrous DMF/Chloroform (3/2 v/v) to solubilize the lipid initiator. A DMF solution (5 mL)
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5 containing predetermined amount of Bn-His-NCA was added to the PE-NH₂ solution using a
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7 transfer needle under nitrogen, and the reaction mixture was allowed to proceed for 72 h under
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9 nitrogen atm. After polymerization, the reaction solution was concentrated under high vacuum,
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11 the product precipitated in cold diethyl ether, and dried under vacuum to yield PE-p(Bn-His)₄₀.
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13 For the de-protection of the benzyl groups, a round-bottomed flask was charged with a solution
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15 of PE-p(Bn-His)₄₀ (100 mg) in trifluoroacetic acid (10 mL). To this was added a 4-fold molar
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17 excess of a 33 wt% solution of hydrobromic acid (HBr) in acetic acid in cold diethyl ether to
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19 precipitate PE-p(His)₄₀. The obtained pale yellow solid was re-dissolved in 10 mL of DMF and
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21 further purified by dialysis against deionized water. Water was removed by freeze-drying to
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23 yield a white solid product. ¹H NMR (400 MHz, DMSO d₆) δ (ppm): 0.96–1.63 (br, CH₃, CH₂),
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25 2.3 (br, CH₂C=O–O), 3.01 (d, nH, CH₂), 3.95 (t, nH, CH), 6.87 (br, nH, Ar-H), 6.97 (br, nH, Ar-
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27 H), 8.10 (br, nH, NH), and 9.35 (br, Ar-NH) ppm. *M_n* = 6800 (by GPC); Đ = 1.24 (by GPC).
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35 **Synthesis of chain transfer agent (CTA) for reversible addition-fragmentation chain**
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37 **transfer (RAFT) polymerization.** Trithiocarbonate CTA, S-1-dodecyl-S'-(α,α'-dimethyl-α''-
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39 acetic acid) trithiocarbonate bearing acid functionality was synthesized according to the
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41 literatures.²³ In brief, 4.04 g of dodecanethiol (20 mmol), 10 mL of acetone, and 0.26 g of
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43 tetrabutyl ammonium bromide (0.8 mmol) were added to a 50-mL flask, and nitrogen gas was
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45 bubbled through the solution for 30 min at 10 °C. An aqueous solution of 1.68 g of 50 wt %
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47 sodium hydroxide (21 mmol) was subsequently slowly added at temperature below 10 °C. After
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49 stirring for 15 min, a carbon disulfide solution in acetone was added in a drop-wise manner (CS₂:
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51 1.525 g, 20 mmol; acetone: 2.015 g, 34.5 mmol). The system was stirred for another 15 min prior
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53 to the addition of 2.4 mL of chloroform (30 mmol) and 8 g of 50 wt % sodium hydroxide (100
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mmol) at temperature below 10 °C. The ice bath was removed 30 min later, and the reaction was allowed to proceed for 12 h before the addition of 30 mL of distilled water and 5 mL of hydrochloric acid (6.8 M). After 30 min, the system was distilled under reduced pressure to remove the volatile solvents, resulting in the appearance of a yellow precipitate which was collected by filtration. The precipitate was dissolved in 100 mL of isopropanol under strong stirring, and the undissolved residue was removed by filtration. The filtrate was subsequently distilled under reduced pressure to remove isopropanol, and the remaining residue was recrystallized in hexane and dried in vacuum for one day. At the end, 4.3 g of trithiocarbonate CTA was obtained as pale yellow solid (Yield = 45%). ^1H NMR (400 MHz, CDCl_3) δ (ppm) = 0.87 (t, 3H, $\text{CH}_3\text{C}_{10}\text{H}_{20}\text{CH}_2-$), 1.26–1.71 (m, 20H, $\text{CH}_3\text{C}_{10}\text{H}_{20}\text{CH}_2-$), 1.72 (s, 6H, $-\text{S}(\text{CS})\text{S}-\text{C}(\text{CH}_3)_2-\text{COOH}$), 3.28 (t, 2H, $\text{CH}_3\text{C}_{10}\text{H}_{20}\text{CH}_2-$). ^{13}C NMR (100 MHz, CDCl_3) δ (ppm) = 14.8, 22.8, 23.2, 28.9, 30.0, 30.3, 31.1, 32.5, 34.3, 55.1, 172.5, 220.3.

Synthesis of alkyne-terminated CTA. Alkyne-terminated S-1-dodecyl-S'-(α,α' -dimethyl- α'' -acetic acid) trithiocarbonate (CTA-alkyne) was synthesized by esterification of S-1-dodecyl-S'-(α,α' -dimethyl- α'' -acetic acid) trithiocarbonate and propargyl alcohol. S-1-Dodecyl-S'-(α,α' -dimethyl- α'' -acetic acid) trithiocarbonate (2 g, 5.5 mmol), EDC-HCl (2.12 g, 11 mmol), and DMAP (0.067 g, 0.55 mmol) were mixed in 20 mL of dry CH_2Cl_2 . Propargyl alcohol (0.635 mL, 11 mmol) was added dropwise to the mixture at 0 °C, and the resulting transparent yellow solution was stirred at room temperature for 48 h. The mixture was subsequently washed three times with distilled water. The ester was further purified with silica column chromatography (eluent: a mixture of petroleum ether and ethyl acetate with a volume ratio of 1:1), and the CTA-alkyne was obtained as a pale yellow liquid (Yield = 51%). ^1H NMR (400 MHz, CDCl_3) δ (ppm) = 0.87 (t, 3H, $\text{CH}_3\text{C}_{10}\text{H}_{20}\text{CH}_2-$), 1.26–1.71 (m, 20H,

CH₃C₁₀H₂₀CH₂–), 1.72 (s, 6H, –S(CS)S–C(CH₃)₂–COOH), 3.28 (t, 2H, CH₃C₁₀H₂₀CH₂–). ¹³C NMR (100 MHz, CDCl₃) δ (ppm) = 14.8, 22.8, 23.2, 28.9, 30.0, 30.3, 31.1, 32.5, 36.3, 53.4, 56.1, 76.2, 78.3, 172.5, 221.2.

Synthesis of p(NIPAM) via RAFT polymerization using CTA–alkyne. A Schlenk tube containing a reaction mixture of NIPAM (1.1760 g, 10.4 mmol), CTA–alkyne (66.1 mg, 0.1 mmol), and AIBN (3.344 mg, 0.02 mmol) in DMF (4 mL) was degassed by three freeze–pump–thaw cycles, and the solution was stirred at 70 °C under N₂ for 24 h. Precipitation in ethyl ether twice yielded alkyne-terminated p(NIPAM) [p(NIPAM)_n–alkyne] (n = 25, 40, 60) as a white powder. ¹H NMR (400 MHz, CDCl₃) δ (ppm) = 0.98 (t, 3H, CH₃C₁₀H₂₀CH₂–), 1.28 (br, CH(CH₃)₂), 1.30–1.96 (br, 20H, CH₃C₁₀H₂₀CH₂–), 2.52 (br, CH₂), 3.47 (br, CH), 3.94 (br, CH), 4.40 (m, 2H, OCH₂), 6.21 (br, NH). ¹³C NMR (100 MHz, CDCl₃) δ (ppm) = 14.08, 20.9, 21.6, 23.1, 23.81, 23.1, 28.9, 30.3, 30.9, 31.7, 32.5, 34.1, 39.4, 40.3, 41.3, 45.6, 52.8, 67.5, 174.1, 174.5, 221.7.

Synthesis of azide-functionalized PE. Lecthin PE (0.2 g, 0.25 mmol) and succinimidyl 5-azidovalerate (0.028 mmol) were dissolved in chloroform (14 mL) in a 100-mL round-bottom flask with a magnetic stirrer. A solution of TEA (54 mg, 0.67 mmol) in 2 mL of chloroform was added to the reaction mixture and stirred at 30 °C for 2 h. The reaction mixture was subsequently washed with water (3 × 30 mL). The solvent was removed under reduced pressure and flash column silica gel chromatography was performed using an 80:20 mixture of chloroform: methanol as an eluent for the separation of azide-terminated lipids (PE–N₃) with yield 64%.

Synthesis of PE–p(NIPAM)₄₀–FA conjugate. Together was mixed 0.07 g (0.1 mmol) of PE–N₃ with 10.0 mL of CHCl₃, 0.41 g of p(NIPAM)₄₀–alkyne (*M*_n = 4100, 0.1 mmol), and 0.5

mol% of $\text{Cu}(\text{PPh}_3)_3\text{Br}$. The reaction was allowed to proceed for 24 h under inert atmosphere at room temperature. The click conjugation between the $\text{p}(\text{NIPAM})_{40}$ and PE was verified the complete disappearance of azide stretching in the FT-IR spectrum at 2230 cm^{-1} . The reaction mixture was passed through silica gel to remove the catalyst and recrystallized in *n*-hexane to yield a white powder. Aminolysis removal of the thiocarbonyl moiety was accomplished as previously described.²⁴ $\text{PE-p}(\text{NIPAM})_{40}$ was dissolved in chloroform and stirred for 2 h under nitrogen atmosphere in the presence of *n*-hexylamine and tributylphosphine. The resulting thiol-terminated $\text{PE-p}(\text{NIPAM})_{40}\text{-SH}$ precipitated as a white powder in cold ether, and was dried under reduced pressure. Its identity was confirmed by the complete disappearance of the RAFT entity at 308 nm by UV-Vis. For the conjugation of FA moiety via click reaction, FA was subject to esterification to yield allyl folate according to previous literature.²⁵ The resulting allyl folate was dissolved in DMF and a solution of $\text{PE-p}(\text{NIPAM})_{40}\text{-SH}$, and TEA dissolved in DMF was added drop-wise. The solution was stirred at room temperature for 12 h under nitrogen atmosphere. The product was precipitated in ice-cold ether twice, and the FA-conjugated lipopolymer ($\text{PE-p}(\text{NIPAM})_{40}\text{-FA}$) was dissolved in DMF and dialyzed for 6 h using a membrane with molecular weight limit of 1000 Da, and then was lyophilized.

Formulation and fabrication of dual-responsive NCs. NCs consisting of $\text{PE-p}(\text{His})_{40}$, $\text{PE-p}(\text{NIPAM})_{40}\text{-FA}$ and PC were fabricated by simultaneous heating-cooling followed by dialysis. Initially, a dry lipid film (20 mg) consisting of $\text{PE-p}(\text{His})_{40}$, $\text{PE-p}(\text{NIPAM})_{40}\text{-FA}$, and PC was prepared by rotary evaporation of 20 mL of chloroform/ethanol (6:4) solution. The weight ratio of $\text{PC/PE-p}(\text{His})_{40}\text{/PE-p}(\text{NIPAM})_{40}\text{-FA}$ was modulated to 50/25/25, 60/20/20, 70/15/15, 80/10/10, and 90/5/5, respectively. The formulated film was further dried under high vacuum for at least 4 h to remove solvent traces. 10 mg of film was dissolved in 8 mL mixed

solvent of ethanol and DMSO (5:1 v/v), then 2 mL water was added drop-wise into the solution, and the resulting solution was heated to 50 °C for 1 h. The clear solution mixture was then allowed to cool slowly at a rate of 1 °C min⁻¹ in an automated thermal-cycler to 25 °C. The resulting solution was transferred into a dialysis membrane (molecular weight cut-off (MWCO) of 1000) at 25 °C and dialyzed against 10 mM PBS, pH 7.4. The outer phase was replaced with fresh buffer solution at 1, 2, 4, 6, and 12 h. After 24 h, the polymers inside the membrane were self-assembled into mixed vesicular nanosphere (VNS). The solution was subsequently lyophilized after filtering through an 800 nm syringe filter. The yield (wt %) of VNSs was calculated by weighing the lyophilized VNS powder.

To obtain photosensitizer-loaded hybrid VNSs, 4 mL aliquots of ethanol/DMSO (5:1 v/v) was used to dissolve Ce6 (20 mg) and dehydrated film (10 mg) and then combined; for a preparation of VNSs, the resulting solution was heated to 50 °C for 1 h and cooled slowly at a rate of 1 °C min⁻¹ to 25 °C in an automated thermal-cycler and then dialyzed against 10 mM PBS, pH 7.4, at room temperature for 24 h. The final mixed VNSs were used after filtration through an 800 nm syringe filter (58% yield).

For quantitative analysis of drug encapsulation, aliquots of the drug-loaded VNS solution were lyophilized and dissolved in 2 mL of DMSO and analyzed using UV-Vis spectroscopy. The characteristic absorbance of Ce6 (663 nm) was recorded and compared to five point standard curve of Ce6 from 0 to 100 mg/mL in DMSO. The percentages of DLC and DLE were calculated using the following equations:

$$\text{DLC (\%)} = \frac{\text{weight of the drug in the VNS}}{\text{weight of drug loaded VNS}} \times 100$$

$$\text{DLE (\%)} = \frac{\text{weight of the drug in the VNS}}{\text{weight of the drug for the preparation of drug loaded VNS}} \times 100$$

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Cell culture. KB cells were cultured in RPMI 1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) and 1% antibiotics at 37 °C in a humidified atmosphere of 5% CO₂. Human normal skin fibroblast CDD-986Sk cells (Korean Cell Line Bank, Seoul, Korea) were cultured in IMEM medium (Gibco) containing 10% FBS and 1% antibiotics. Cells were sub-cultured twice per week.

Cytotoxicity of formulated VNSs. KB and CDD-986Sk cells were seeded into 96-well plates. For starvation, the cells were incubated in RPMI or IMEM medium containing 0.1% FBS for 24 h. After removing the medium, the cells were washed with phosphate-buffered saline (PBS). Next, 100 µL of fresh serum-free RPMI or IMEM medium containing various concentrations of polymer (0-100 µg/ml) was added to each well. The cells were then incubated at 37 °C for 24 h. Finally, the KB or CCD-986Sk cells were washed twice with PBS and the cytotoxicity was measured using the MTT assay.

Dark toxicity of Ce6 or Ce6@VNS (Ce6@VNS). KB cells were seeded into a 96-well plate and treated with various concentrations of Ce6 or Ce6@VNS (0.0–1.0 µM) at both pH 6.6 and 7.4 in serum-free medium for 3 h. After removing the medium, the cells were washed twice with PBS. To each well, 100 µL of the RPMI medium containing 10% FBS was added and the cells were incubated for additional 24 h. Cell viability was measured using the MTT assay.

Intracellular uptake of Ce6 or Ce6@VNS. KB cells were seeded into 96-well plates at a density of 2×10^4 cells per well and incubated for 24 h. The cells were washed twice with PBS and incubated for 3 h with serum-free medium containing 0.1 µg/mL of Ce6 or Ce6@VNS at different pH (pH 6.6 or 7.4) or culture temperature conditions (25 or 37 °C). After washing twice

with PBS, the cells were solubilized in 50 μ L of cell lysis buffer (Gen DEPOT, USA). Ce6 fluorescence showed excitation at 407 nm and emission at 664 nm using an Infinite M200 pro microplate reader (Tecan, Switzerland).

Fluorescence microscope. Intracellular Ce6 was observed using fluorescence microscopy. Approximately 2×10^5 cells were seeded onto a cover glass in a 6 well plate. The cells were then treated with 0.5 μ g/mL Ce6 or Ce6@VNS for 3 h at different pH (pH 6.6 or 7.4) or culture temperature conditions (25 or 37 $^{\circ}$ C) in serum-free media. Afterwards, the medium was discarded and the cells were washed with PBS. The cells on the cover glass were observed using fluorescence microscope equipped with a 590 nm excitation filter and a 615 nm filter to detect Ce6 fluorescence.

Photodynamic therapy. KB cells were seeded into a 96 well plate and cultured for 24 h. The cells were then washed twice with PBS, and 100 μ L of serum-free medium, pH 6.6 or 7.4, containing 0.1 μ g/mL Ce6 or Ce6@VNS was added to each well. The cells were then incubated for 3 h at either 25 or 37 $^{\circ}$ C, the medium removed, and the cells washed twice with PBS. Next, 100 μ L of fresh serum-free RPMI was added and the cells exposed to an expanded homogenous beam of 664 nm radiation (SH Systems, Korea) at a dose of 1.0 J/cm² measured by a photo-radiometer (DeltaOhm, Italy). After irradiation, the cells were incubated for an additional 24 h. Cell viability was determined using the MTT assay.

Folic acid inhibition. KB cells were seeded into a 96 well plate and cultured for 24 h. After washing the cells with PBS, 100 μ L of serum-free RPMI media containing folic acid (10 μ g/mL) was added to each well and the cells were incubated for 1 h at pH 6.6 and 37 $^{\circ}$ C. The medium was then discarded and 100 μ L of serum-free RPMI media, pH 6.6, containing folic

acid (10 $\mu\text{g/mL}$) and Ce6 or Ce6@VNS (0.1 $\mu\text{g/mL}$) were added to each cell and further incubated for 1 h at 37 $^{\circ}\text{C}$. Intracellular Ce6 uptake or PDT was done as described above.

ROS assay. After irradiation, ROS generation induced by Ce6 was measured using the DCFH-DA method.²⁶ In brief, the fluorogenic substrate DCFH-DA, a cell permeable dye, oxidized to the highly fluorescent DCF when exposed to ROS, which was used to monitor intracellular ROS generation. The cells were treated with Ce6 or Ce6-apsulated VNS (0.1 $\mu\text{g/mL}$) containing DCFH-DA at a final concentration of 20 μM in RPMI media (free of phenol red) at different pH (6.6 or 7.4) and incubated at 25 or 37 $^{\circ}\text{C}$ for 3 h. Next, the medium was removed and the cells were washed twice with PBS, and 100 μL of RPMI media (free of phenol red) was added to each well. After irradiation at 1.0 J/cm^2 (664 nm), ROS generation was based on fluorescence intensity measurements at an excitation of 485 nm and emission of 535 nm using an Infinite M200 pro microplate reader (Tecan).

Flow cytometry analysis. To evaluate apoptotic and necrotic cells, FITC-annexin V and PI were used, respectively. Following PDT, the uptake of these two reagents was assessed using a FACScan flow cytometer. KB cells were treated with Ce6 or Ce6@VNS (0.1 $\mu\text{g/mL}$) in serum-free RPMI medium at pH 6.6. The cells were incubated at 25 or 37 $^{\circ}\text{C}$ for 3 h, the medium discarded, the cells washed twice with PBS, and 100 μL of fresh serum-free medium added to each well. The cells were then exposed to a dose of 1.0 J/cm^2 of radiation. Cells were harvested by trypsinization and washed with PBS. The cells were resuspended in binding buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES] at pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , and 1.8 mM CaCl_2) containing FITC-annexin V (1 $\mu\text{g/mL}$) and PI (10 $\mu\text{g/mL}$), and further incubated for 15 min at room temperature (25 $^{\circ}\text{C}$) in the dark. The cells

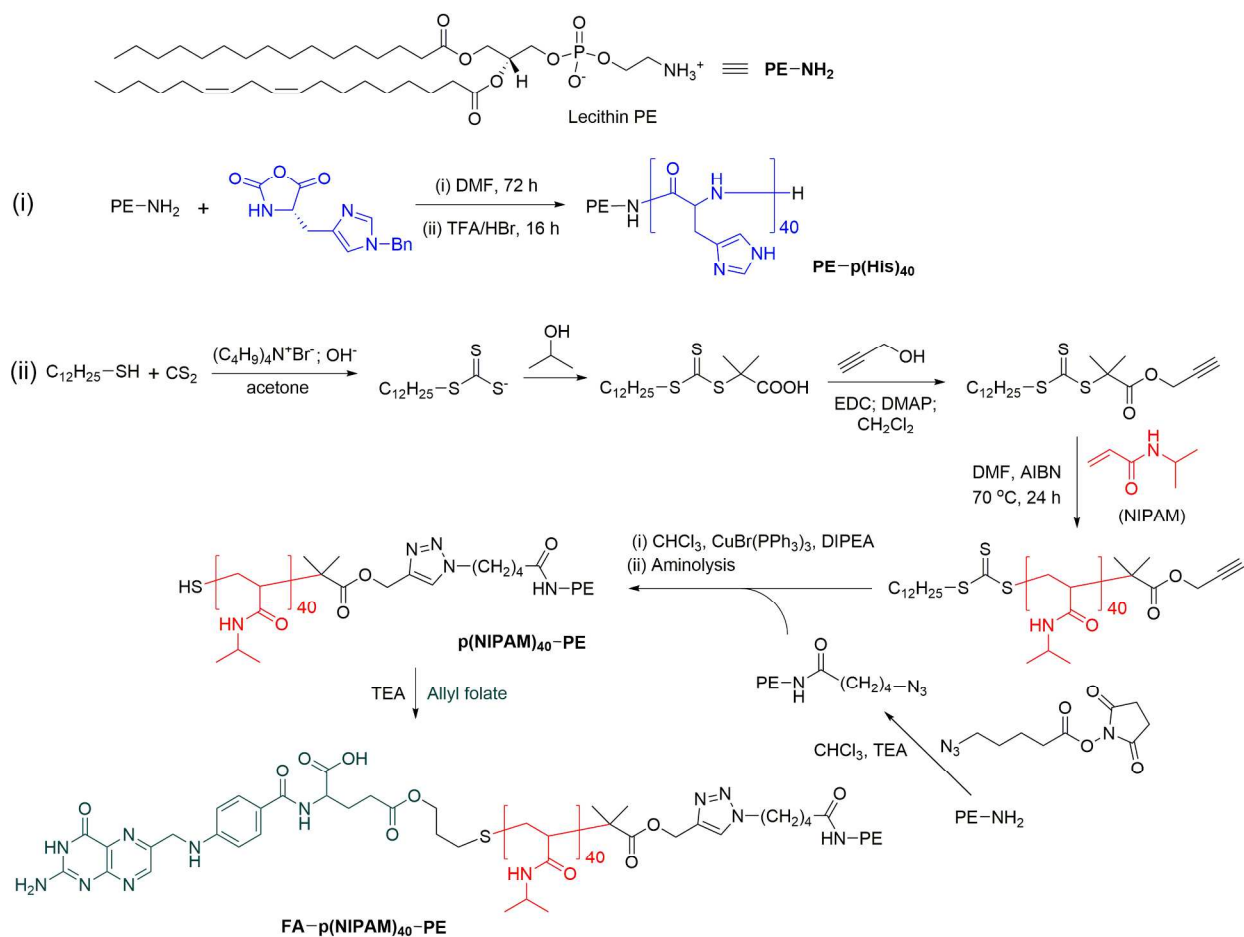
were immediately analyzed using a FACScan flow cytometer (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA).

Statistical data analysis. Statistical analyses were performed using analysis of variance (ANOVA). All experiments were performed at least three times, and data are presented as mean $\pm$ standard error of the mean (SEM).

RESULTS AND DISCUSSIONS

Synthesis of PE-p(His)₄₀ and PE-p(NIPAM)₄₀-FA conjugates. The synthetic procedures of PE-p(His)₄₀ and PE-p(NIPAM)₄₀-FA conjugates are summarized in Scheme 1. A high composition of PC and PE was simultaneously extracted from soybean lecithin by using a mixed solvent consisting of ethanol and chloroform.²² For the synthesis of PE-p(His)₄₀ the ring-opening polymerization (ROP)¹⁰ of Bn-His-NCA was performed using PE-NH₂ as an initiator. Analyses of the resulting PE-p(Bn-His) conjugate by using NMR and gel permeation chromatograph showed that the block size of Bn-His was about 40 units. The benzyl side chains were then deprotected by treating PE-p(Bn-His)₄₀ with HBr/AcOH in trifluoroacetic acid, and the polymer was characterized by NMR (Figure S1 of supporting information (SI)). The peak integral ratio of the protons of PE (δ = 0.9–1.6 ppm) and imidazole (a+b+c+d) in the peptide block (δ 7.2 to 8.5 ppm) was used to calculate the number average molecular weight (M_n), confirming that there was no loss of the repeating histidine units during the deprotection.

The FA conjugated PE-p(NIPAM)₄₀ was synthesized by combining the RAFT polymerization with the azide-alkyne and thiol-ene click reactions (Scheme 1). Firstly alkyne terminated RAFT chain transfer agent (CTA)²⁷ was used for the polymerization of NIPAM to yield p(NIPAM)₄₀ terminated with alkyne moiety. The resulting compound was subject to click



Scheme 1. Synthesis routes of (i) PE-p(His)_{40} and (ii) $\text{PE-p(NIPAM)}_{40}\text{-FA}$.

reaction with PE modified with azide group. The aminolysis of RAFT CTA fragment and the subsequent removal of the catalyst by column filtration and thiocarbonyl moieties yielded $\text{PE-p(NIPAM)}_{40}\text{-SH}$ hybrids. Figure 1(A) shows the FT-IR spectra of alkyne-terminated p(NIPAM)_{40} , azide-functionalized PE, and PE-p(NIPAM)_{40} hybrids. In the synthesis of PE-p(NIPAM)_{40} hybrids, the azide stretching of PE-N_3 at 2230 cm^{-1} disappeared in the PE-p(NIPAM)_{40} spectra. Additionally, the single ^{31}P NMR chemical shift at $\delta = -1.3\text{ ppm}$ confirms the successful conjugation of the lipids in p(NIPAM)_{40} . Hay et al.²⁸ reported a strong isotropic resonance at 1.33 ppm in the ^{31}P NMR of dimyristoylphosphatidylethanolamine- p(NIPAM) hybrids obtained by carbodiimide coupling strategy. The removal of

thiocarbonyl moiety was confirmed by comparing the UV–visible spectra of CTA, p(NIPAM)₄₀, PE–p(NIPAM)₄₀, and PE–p(NIPAM)₄₀–SH, shown in Figure 1(B). The RAFT entity (typical absorbance at 308 nm) completely disappeared after aminolysis.

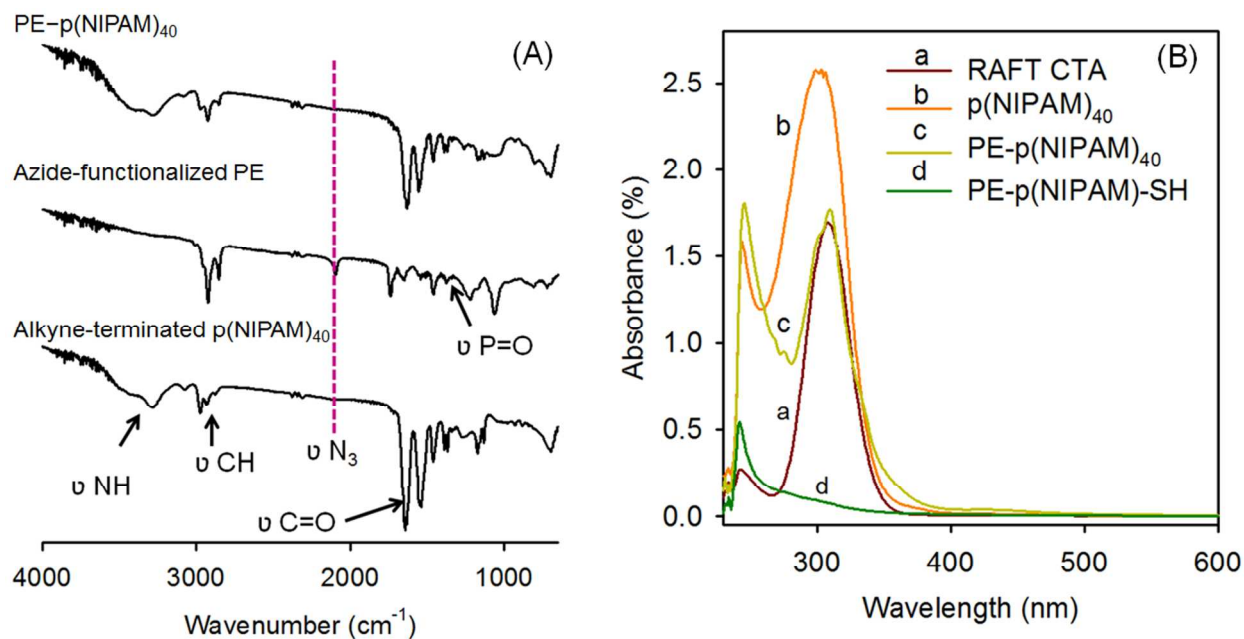


Figure 1. (A) FT–IR spectra of alkyne-terminated p(NIPAM)₄₀, azide-functionalized PE and PE–p(NIPAM)₄₀ hybrids, and (B) UV–visible spectra of RAFT CTA, p(NIPAM)₄₀, PE–p(NIPAM)₄₀ and PE–p(NIPAM)₄₀–SH.

The resulting ω–functional thiol group was utilized for the conjugation of allyl folate as a targeting ligand by thiol–ene click reaction. The folic acid-tethered PE–p(NIPAM)₄₀ lipopolymers were purified by precipitation and lyophilization to remove the nonconjugated allyl folate. ¹H NMR (Figure S2 of SI) spectroscopy was used to confirm the folic acid conjugation in the PE–p(NIPAM)₄₀–SH lipopolymer. The chemical shift at δ = 8.67 ppm shows the presence of a pteridine ring of the allyl-folate in the PE–p(NIPAM)₄₀–FA conjugate.²⁵ The allyl proton of allyl folate at δ = 5.29 ppm and 5.64 ppm (–CH=CH₂) disappeared after the thiol–ene reaction,

and the proton signals near $\delta = 6\text{--}7.5$ ppm of folate merged with the N–H proton of the p(NIPAM)₄₀ repeating units. The spectroscopic data reveal the successful attachment of the folic acid ligand to the lipopolymer hybrids. Note that the removal of CTA end-groups is one of the major issues in the RAFT polymerization, since their decomposition produces malodorous sulfur-containing materials.²⁴ In this work, we converted the CTA fragment to thiol group to attach folate for enhance the targetivity of NCs.

Fabrication of NCs. New generation liposomal formulations like PEGylated liposomes may extend blood circulation time, vary drug distribution in the body, and hence reduce the possible side effects related to the drugs; however, they displayed severe and harmful toxic effects on normal organs and tissues in clinical trials, most probably relating to PEGylation. Moreover, most of the liposomes and liposomal formulations were developed for drug delivery vehicles. As a means of developing a new pH/temperature responsive and targeted lipopolymer PDT formulation that may overcome the drawbacks of the conventional liposomal formulations, we have chosen a system consisting of lecithin oriented PC, PE–p(His) conjugate where p(His) block shows pH response, and FA conjugated PE–p(NIPAM) where p(NIPAM) block shows temperature response.

NCs consisting of PC, PE–p(His)₄₀, and PE–p(NIPAM)₄₀–FA were fabricated by simultaneous heating-cooling followed by dialysis. The dried hybrid films fabricated by controlling the weight ratio of PC/PE–p(His)₄₀/PE–p(NIPAM)₄₀–FA to 50/25/25, 60/20/20, 70/15/15, 80/10/10, 90/5/5 were dissolved in mixed solvent of ethanol and DMSO, then water was added into the solution. The turbid solutions were heated to 50 °C for 1 h to obtain clear solution mixtures, then were allowed to cool slowly at a rate of 1 °C min^{–1}. The resulting solutions were subjected to dialysis and lyophilization to yield VNS powders. The size and

morphology of the fabricated hybrid vesicles were characterized by TEM (Figure 2). The average particle size is around 100 nm. The beneficial properties of liposomes as drug carriers led to the development of fabrication methods such as extrusion, high-energy sonic fragmentation, high-pressure homogenization, solvent injection, freeze-thaw cycling, de/rehydration, reverse evaporation, electro-formation, solid film dehydration, detergent dialysis, etc. Different preparation techniques result in different types of liposomes, categorized by lamellarity (multi-/unilamellar vesicles) and size range [small (≤ 100 nm), intermediate (100–250 nm), large (≤ 250 nm), or giant ($> 1 \mu\text{m}$)].²⁹ Surface charge of vesicles is classified into anionic, cationic, or neutral base on the chemical structure of lipid used. The lecithin PC is one of the typical charge neutral phosphatidylcholines.

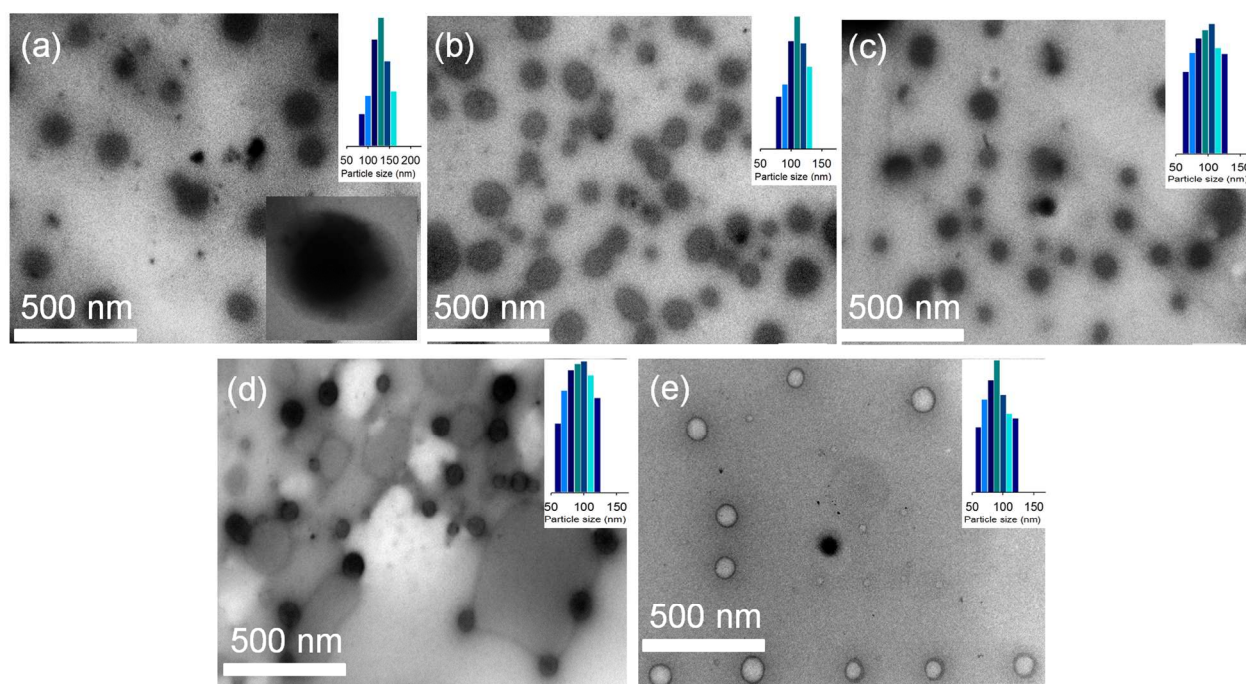


Figure 2. TEM images of nanocarriers fabricated by PC, PE-p(HIS)₄₀, and PE-p(NIPAM)₄₀-FA mixtures composed of different weight ratios: (a) 50/25/25, (b) 60/20/20, (c) 70/15/15, (d) 80/10/10 and (e) 90/5/5.

According to the sizes of the NCs consisting of PC, PE-p(His)₄₀ and PE-p(NIPAM)₄₀-FA, the vesicles obtained here can be categorized as small range. The size of the hybrid VNSs in different formulations was also confirmed by DLS (Table 1). As the relative composition of PC increases from 50% to 90%, the average particle size decreases from 125 nm to 97 nm. Note that the particle size distribution is also reasonably narrow. In fact the undesirable and irreproducible distribution of liposomes fabricated by pure phospholipids is one of the critical factors of importance in industrial applications. The lamellarity of the NCs is ambiguous, because of the participation of the long p(His) and p(NIPAM) chains in forming vesicles. In fact, the vesicles are quite different from those fabricated by pure PC, so that they are much like micelles (*vide infra*).

Table 1. Compositions, size and physical properties of cationic dual responsive nanocarriers.

Sample	Composition	Average particle size ^[a] (nm)	ζ potential (mV) at pH 6.6	Ce6	Ce6
	PC/ PE–p(His) ₄₀ /PE– p(NIPAM) ₄₀ –FA			DLC ^[b] (%)	DLE (%)
A	5.0/2.5/2.5	125±8	+24.3	8.67	45
B	6.0/2.0/2.0	119±5	+18.1	7.20	38
C	7.0/1.5/1.5	108±10	+12.2	5.91	32
D	8.0/1.0/1.0	100±6	+10.3	3.21	25
E	9.0/0.05/0.05	97±10	+3.9	2.86	19

^[a] Determined by DLS. ^[b] Drug (Ce6) loading content and drug loading efficiency.

Careful investigation of the TEM images of the NCs in Figure 2 shows an interesting transformation of the lipid hybrid NCs from VNSs to liposome vesicles. Conjugating polymer chains into the hydrophilic head leads to the creation of vesicles different from uni- and multilamellar phospholipid vesicles (liposomes) that are spherical soft-matter particles consisting of one or more bilayer membrane(s), encapsulating a volume of aqueous medium. The VNSs

obtained by PC, PE-p(His)₄₀, and PE-p(NIPAM)₄₀-FA with 50/25/25 ratio are semisolid spheres, wherein the aqueous lipid phase is replaced by macromolecular constituents. Figure 3 illustrates the transformation of the VNSs according to the variation of composition. Considering both inside and outside of the phospholipid membranes are covered by hydrophobic p(His) and hydrophilic p(NIPAM) constituents, the aqueous volume is reduced to a minimum, giving rise to steric interactions between neighboring vesicles and thus to a semisolid consistency. As a result the VNSs in this condition are much like liposome gels, wherein hydrophobic polymer forms embedding and hydrophilic polymer form encapsulating matrices.

The macromolecular constituents originated packing density decreases accordingly as the amount of them decreases. As expected, minimizing the amount of PE-p(His)₄₀, and PE-p(NIPAM)₄₀-FA conjugates during the micellization, say PC, PE-p(His)₄₀, and PE-p(NIPAM)₄₀-FA in 90/5/5 ratio, resulted in predominantly hollow vesicles (Figure 2(e)). On the other hand stable VNS could not be achieved by increasing the amount of PE-p(His)₄₀, and PE-p(NIPAM)₄₀-FA conjugates higher than 50%, due to severe steric interactions preventing phospholipids and their fragments from spontaneous self-assembly.

Electrostatic interaction is an important secondary force affecting the structure, stability, and function of liposomes. In addition to particle size and its distribution and shape surface charge plays a key role in the cellular uptake of liposomes, since the uptake of them by cells is proceeded by a two-step process: first, a binding step on the cell membrane and second, the internalization step. Furthermore, surface charge affects not only the stability of dosage forms and their release rate but also their circulation in the blood value stream and absorption into body membranes. Table 1 shows zeta potential (ξ) values of the VNSs that are associated with a realistic magnitude of surface charge. The ξ value decreases monotonously from +24.3 (PC/PE-

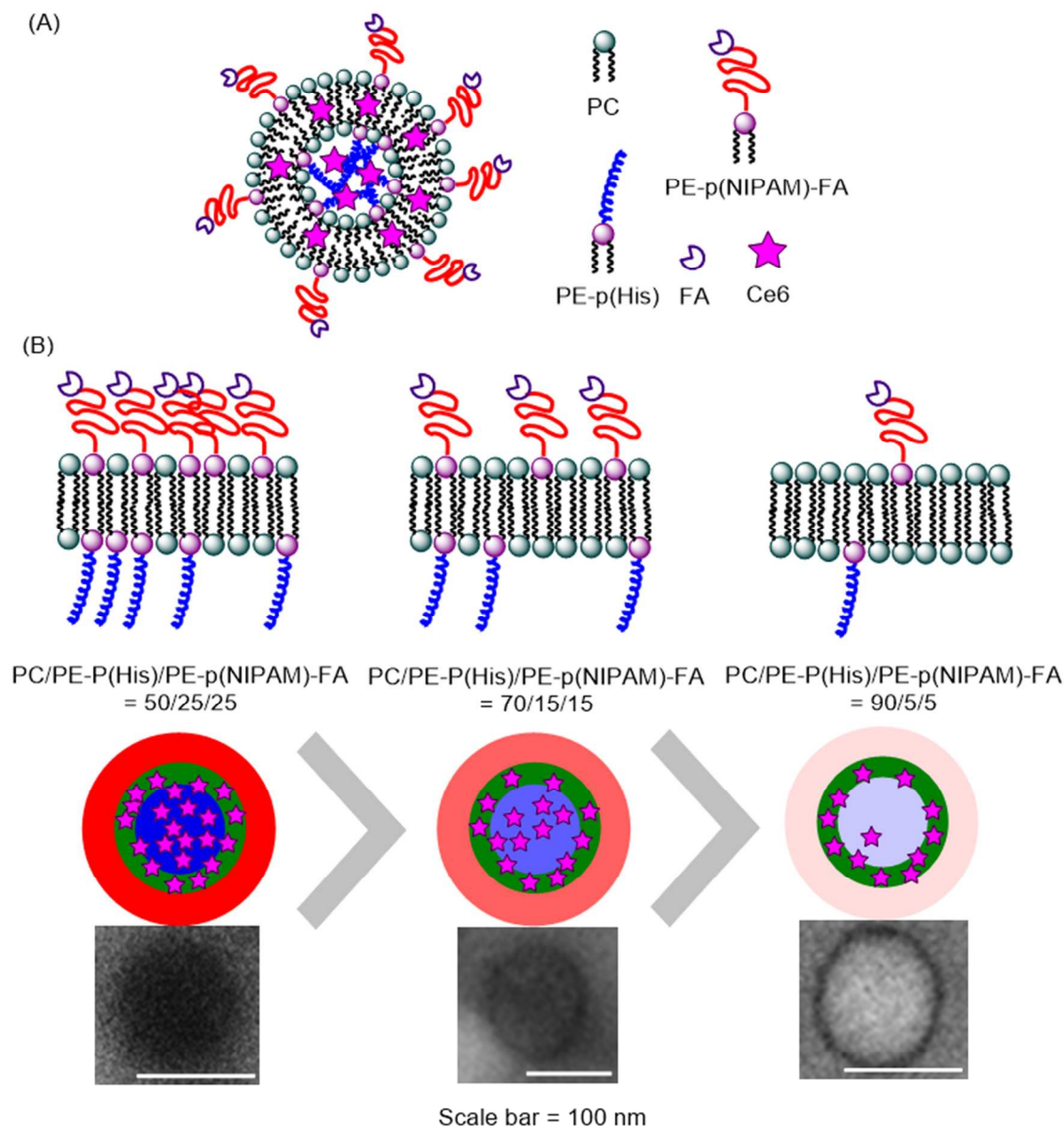


Figure 3. (A) Diagrammatic representation of VNS bearing Ce6 fabricated by PC, PE-p(His)₄₀, and PE-p(NIPAM)₄₀-FA mixtures; (B) morphological transition of VNS from semisolid VNSs to liposomes by modulating relative composition of PC, PE-p(His)₄₀, and PE-p(NIPAM)₄₀-FA.

p(His)₄₀/PE-p(NIPAM)₄₀-FA = 50/25/25) to +3.9 (PC/PE-p(His)₄₀/PE-p(NIPAM)₄₀-FA = 90/5/5), as the PC ratio increases, demonstrating the nano-suspension stability of VNSs decreases as the PC ratio increases. It is interesting to note that all the vascular structures are

characterized by cationic surface charge. Positive ξ value is important for drug delivery since it can facilitate effective adhesion to cancer cell surfaces, which are usually charged negatively due to the translocation of negatively charged constituents of the inner layer of the cell membrane (e.g., phosphatidylserine, anionic phospholipids, glycoproteins and proteoglycans)³⁰, prolonging the drug release and enhancing the drug bioavailability in the internal tissue due to the electrostatic interaction.

Conventional liposomes prepared by the self-assembly of neutral phospholipids are structurally unstable, have low drug loading capabilities and tend to leak the internalized drugs through their boundary. Sterically stabilized or PEG-ylated liposomes, so called stealth liposomes, were reported to provide stabilization enhancement and additional protection of the encapsulated substances.^{31,32} Likewise, the VNSs fabricated from the mixture of PC, PE-p(His)₄₀, and PE-p(NIPAM)₄₀-FA are expected to show stealth structure and peculiar functionality, since the pH-sensitive and hydrophobic p(His) chains are embedded inside of the structure and temperature-sensitive p(NIPAM) chains are located outside of the structure. Considering the attractive PDT drug candidate Ce6 is hydrophobic and big molecule, the VNSs are expected to show different DLC and DLE values in encapsulating Ce6. The surface charge of the particles and binding type between the drug and nanoparticles are the important parameters that determine the rate of desorption of the drug in the nanoparticles and drug loading efficiency.^{33, 34}

Table 1 summarizes those values of VNSs obtained from different compositions. The DLC value of VNSs fabricated by PC/PE-p(His)₄₀/PE-p(NIPAM)₄₀-FA mixtures in 50/25/25 and in 90/5/5 ratios is 8.67% and 2.96%, respectively, showing nearly 3-fold difference. The value decreases monotonously as the PC ratio increases. The increase of p(His) chains inside of VNS

may increase the van der Waals interaction and π - π stacking between p(His) fragments and Ce6 molecules. Note that PSs loaded cationic liposomes have high affinity for angiogenic endothelial and tumor cells.^{19, 20} In addition Figure S3 shows the absorption and fluorescence spectra of the Ce6 loaded PC/PE-p(His)₄₀/PE-p(NIPAM)₄₀-FA (50/25/25) VNS. The peak at 663 nm in both spectra shows the drug loading ca of the system. The fluorescence spectra also demonstrate the pH responsive release of Ce6 from the VNS at 37 °C. The enhanced release of Ce6 may occur not only by the pH-induced destabilization but also by the thermoresponsive behavior of our VNS at 37 °C.

Time dependent release of Ce6. The time dependent releases of Ce6 were evaluated at different temperatures and pHs in phosphate buffered saline (PBS, pH 7.4) by using UV-Vis spectroscopy. Note that the absorption maximum of Ce6 appears at 663 nm in solution. The Ce6-loaded VNS fabricated by PC/PE-p(His)₄₀/PE-p(NIPAM)₄₀-FA in 5.0/2.5/2.5 ratio was chosen as a representative sample because it showed the highest DLE and DLC values. The release performance was observed for 50 h at different temperatures and the resulting profiles are shown in Figure 4. Comparing the release of Ce6 at 37 °C (~55% release in 50 h) to that of Ce6 at 25 °C (~15% release in 50 h), it is evident that the release is accelerated around the transition temperature of the p(NIPAM) block. Accordingly the drug release obtained at 41 °C showed a conspicuous initial bursting of Ce6 (~35%) that is comparable to that (~11%) obtained at 37 °C. Thus, the drug release tests at far above LCST of the VNSs may not show any beneficial effects and detailed investigations were not performed at higher temperature than 37 °C. The temperature sensitivity of the VNSs is reflected by variations of the p(NIPAM) block that shows a LCST around our body temperature from the hydrophilic to the hydrophobic state in response to temperature. The hydrophobicity of the p(NIPAM) block induces low solubility of the

polymer in water by breaking the hydrogen bond of the amide functionality in p(NIPAM) chain, destabilizing the VNSs. Therefore, the LCST of p(NIPAM) chains clearly affect the Ce6 release from VNS owing to the thermal transition of p(NIPAM) chain.

Since tumor tissues contain large, acidic outside plasma pH gradients, while normal tissues generally have alkaline outside pH gradients³⁵, the extracellular pH of tumors is significantly lower than that of normal tissues under physiological conditions³⁶. Thus, the pH-responsive vesicles should show improved fusogenic potential to an endosome mimicking anionic membrane at acidic pH. Considering the ξ value of the VNSs of this study is positive under acidic pH conditions and the size of the particle is around 100 nm, the cationic liposomes should adhere to the negatively charged tumor cell membrane and accelerate the intracellular uptake by epithelial cells.³⁷ For the effective PDT, vesicles bearing Ce6 should preferably release Ce6 in the targeted tumor cells. *In vitro* release rates of Ce6 were investigated by a set of time-dependent release experiments at different pHs 37 °C (Figure 4(B)). About 52% of the incorporated Ce6 is released within 50 h at pH 7.4. The release rate is accelerated at acidic pH conditions, thus, the value increases to 58, 73, and 82% at pH 7, 6.6, and 6.0, respectively. The accelerated rate, especially at pH 6.0, might be related to pKa (~ 6.0) of the imidazole side chain of the p(His) block. These data clearly demonstrate the release rate is sensitive to pH due to the pH-induced destabilization of the p(His) block within the VNSs. Because the p(His) block is ionizable under acidic conditions, its swelling behavior greatly depends on the pH due to the ionization-deionization of the imidazole ring of the p(His) block. Under acidic conditions, the p(His) block is ionized and the charged imidazole groups repel each other leading to high swelling.

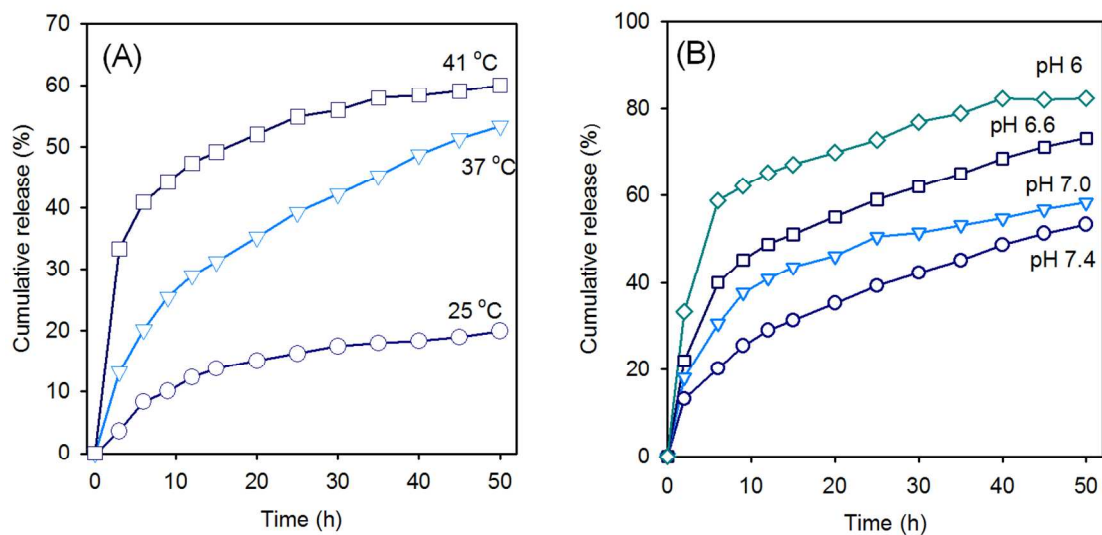


Figure 4. Time-dependent releases of Ce6 from the VNS fabricated by PC/PE-p(His)₄₀/PE-p(NIPAM)₄₀-FA (50/25/25) estimated (A) at different temperatures (pH = 7.4) and (B) at different pH values (temperature = 37 °C).

Cytotoxicity of VNSs fabricated by PC/PE-p(His)₄₀/PE-p(NIPAM)₄₀-FA hybrids.

Above results showed the VNSs fabricated by PC/PE-p(His)₄₀/PE-p(NIPAM)₄₀-FA are characterized by i) stable and small particle size (~ 100 nm) and narrow particle size distribution, ii) positive surface charge, iii) high hydrophobic drug (Ce6) loading efficiency, iv) covalently linked targeting moiety, and v) smartness, in which the drug release is responsive to temperature as well as pH. Accordingly the VNSs of this study have some pros in targeted drug delivery. However, positively charged lipids are not approved by FDA for clinical use³⁸ and in some cases cationic charge is claimed to enhance cytotoxicity³⁹. Positive charge shows a membrane destabilization and concomitantly destructive effect resulting from an interaction of positive charge and negative charge of membrane.⁴⁰ The biocompatibility of the newly formulated VNSs was tested against human fibroblast CCD-986Sk cells and human oral squamous carcinoma KB cells. As shown in Figure 5, the viability of the two kinds of cells was higher than 80% in a

polymer concentration range from 0 to 10 $\mu\text{g/mL}$ over 24 h of cultivation, even though considerable cell cytotoxicity was observed at very high concentrations like 100 $\mu\text{g/mL}$ that is not practical for *in vitro* and/or *in vivo* study. The result reveals that the VNSs show substantial biocompatibility in cell culture and have no acute and intrinsic cytotoxicity against carcinoma (KB) and normal (CCD-986Sk) cells.

The dark toxicity of free Ce6 and Ce6@VNS was also studied using KB cells. As shown in Figure 6, no significant dark toxicity is observed in the presence of less than 1.0 $\mu\text{g/mL}$ Ce6 or Ce6@VNS. Additionally, pH has no effect on cell viability. These results indicate that drug, polymer, and culture conditions have no effect on cell survival.

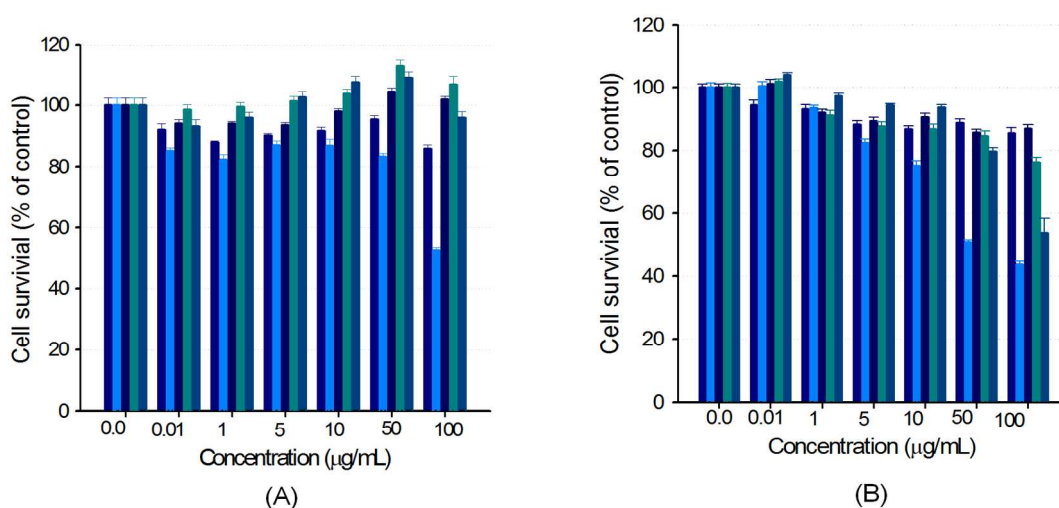


Figure 5. Intrinsic cytotoxicity of VNSs fabricated by PC/PE-p(His)₄₀/PE-p(NIPAM)₄₀-FA mixture in different formulations against (A) CCD986-Sk and (B) KB cells. After starvation for 18 h, the cells were treated with various concentration of polymer micelle. Cell viability was revealed as percent of control. In each concentration PC/PE-p(His)₄₀/PE-p(NIPAM)₄₀-FA ratio is corresponding to 50/25/25, 60/20/20, 70/15/15, 80/10/10 and 90/5/5 in order from the left.

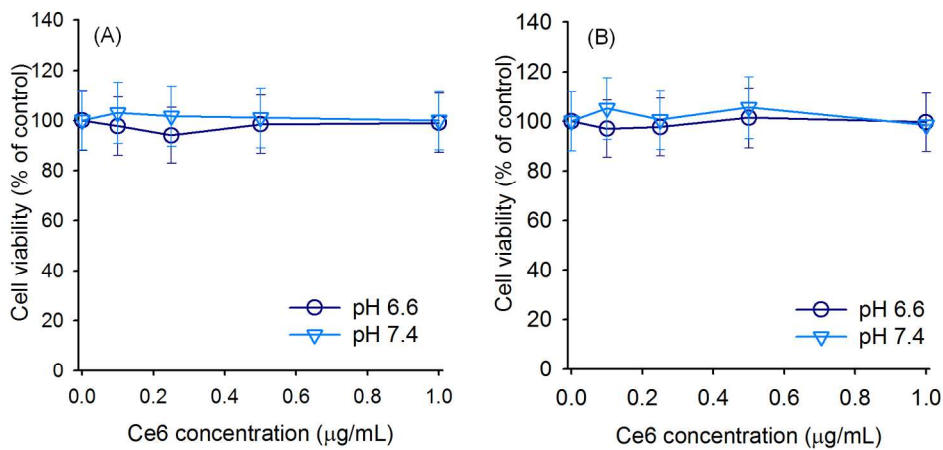


Figure 6. Dark toxicity of (A) free Ce6 and (B) Ce6@VNS (PC/PE-p(His)₄₀/PE-p(NIPAM)₄₀-FA = 50/25/25) against KB cells; KB cells were incubated with 0-1 μg/mL Ce6 or Ce6@VNS in the dark at different pH conditions for 3 h. Cell survival was expressed as the percentage of the control (not exposed to Ce6 or Ce6@VNS) and was determined using the MTT assay.

Intracellular uptake of Ce6 or Ce6@VNS. The uptakes of free Ce6 and encapsulated Ce6 inside of Ce6@VNS in KB cells were also investigated. As shown in Figure 7, the two systems show a dramatic difference in the degree of intracellular uptake of Ce6. Incubating KB cells with 0.1 μg/mL of Ce6 or Ce6@VNS for 3 h at different pH (pH 6.6 or 7.4) and culture temperatures (25 or 37 °C), the amount of free Ce6 inside of the cell is almost negligible; however, considerable amount of encapsulated Ce6 inside of Ce6@VNS is observed inside of the cell. In addition Ce6 molecules are more efficiently delivered inside of the cells at low pH and 37 °C. Note that the KB cell membrane is most probably in acidic condition. Evidently the dramatic increase of intracellular Ce6 delivery must be related with the features of Ce6@VNS: i.e. suitable size (~ 100 nm) and size distribution, stability, cationic surface charge, specificity (or targetability), and smartness responding to pH and temperature. The efficient delivery of Ce6 is a prerequisite condition to perform an efficient PDT.

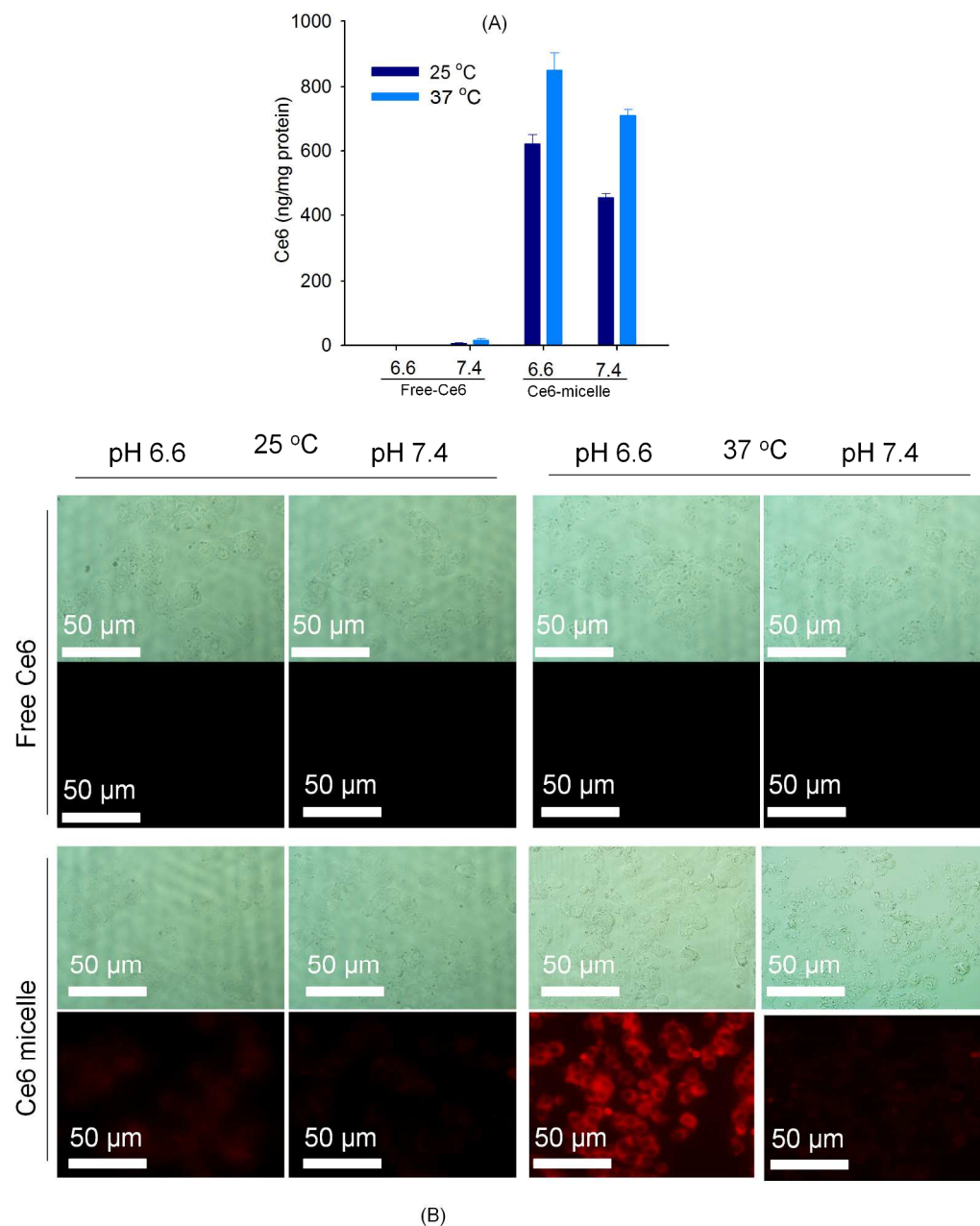


Figure 7. Intracellular accumulation of Ce6 in KB cells at different pH and temperature conditions: (A) comparison of the amount of intracellular Ce6 uptake of free Ce6 with Ce6@VNS (PC/PE-p(His)₄₀/PE-p(NIPAM)₄₀-FA = 50/25/25) and (B) optical and confocal laser scanning microscopic images of KB cells. The KB cells were incubated with 0.1 μ g/mL free Ce6 or Ce6@VNS for 3 h. The amount of intracellular Ce6 was determined fluorometrically.

Photodynamic therapy based on free Ce6 and Ce6@VNS. The phototoxicity of Ce6 or Ce6@VNS (0.1 $\mu\text{g/mL}$ Ce6) against KB cells was investigated. Before irradiation at 1.0 J/cm^2 , serum free RPMI medium with 0.1 $\mu\text{g/mL}$ Ce6 or Ce6@VNS was administered to the cells at pH 6.6 or 7.4, and the KB cells were incubated for 3 h at 25 or 37 $^{\circ}\text{C}$. Figure 8(A) illustrates degrees of cell viability after irradiation. Since the amount of Ce6 delivered inside of the cells was negligible from the incubation with free Ce6 regardless of the incubating conditions, the cell viabilities are higher than 90% in all cases. There were no significant differences in phototoxicity for free-Ce6 at 0.1 $\mu\text{g/mL}$. On the other hand, significant phototoxicity is observed in KB cells treated with Ce6@VNS. Specifically the cell viability records around 30% at pH 6.6 and 37 $^{\circ}\text{C}$, clearly demonstrating the Ce6@VNS system is more beneficial than free Ce6 for PDT to treat tumor cells. In order to get further insights on the pH responsive behavior we have recorded the fluorescence spectra of Ce6-loaded VNS at different pHs: pH 7.4, pH 6.6, pH 6.0, pH 5.0, and pH 4.0 (see Figure S3(B)). The peak intensity of Ce6 increases from pH 7.4 to pH 6.6 and even more to pH 6.0. Further decrease of pH results in decreasing of the peak intensity of Ce6, demonstrating that there exists an optimal pH showing the best efficiency, which is deeply related to the existence of three carboxylic groups in Ce6, providing hydrophobic core holding rather than destabilization at low pH. The peak maxima are also shifted according to pH^{41} : i.e. 667 nm, 663 nm, 661 nm, 650 nm, and 648 nm for pH 7.4, pH 6.6, pH 6.0, pH 5.0, and pH 4.0, respectively.

PDT involves the photosensitization of neoplastic cells and tissues with PSs that can catalyze formation of potentially lethal ROS upon irradiation. The ROS leads to the chemical destruction of any tissues which have both selectively taken up the photosensitizer and have been locally exposed to light. Thus the degree of ROS generation can be a measure of PDT efficacy.

As shown in Figure 8(B), ROS generation is markedly increased in the Ce6@VNS treated group when compared to the free-Ce6 group regardless of culture conditions. As expected, ROS level is especially elevated when Ce6@VNS was treated at pH 6.6 and 37 °C, demonstrating the results from phototoxicity tests are in line with those from ROS generation tests.

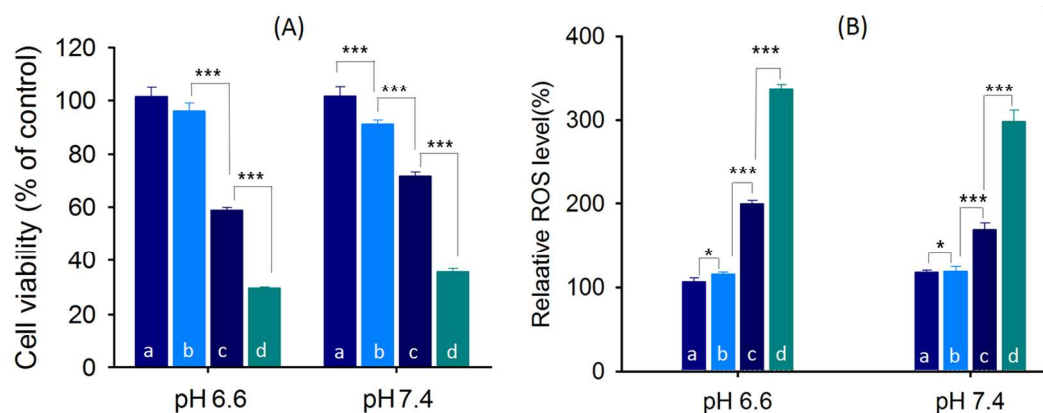


Figure 8. (A) Phototoxicity of free Ce6 (a and b) and Ce6@VNS (PC/PE–p(His)₄₀/PE–p(NIPAM)₄₀–FA = 50/25/25) (c and d) against KB cells irradiated with 1.0 J/cm² light after incubating them with 0.1 µg/mL Ce6 or Ce6@VNS at pH 6.6 or 7.4 and at 25 °C (a and c) or 37 °C (b and d) for 3 h; (B) relative ROS level after photodynamic treatments in the presence of free Ce6 (a and b) and Ce6@VNS (c and d). For the measurement of ROS level KB cells were treated with free Ce6 and Ce6@VNS with DCFH-DA at a final concentration of 20 µM in phenol red serum-free RPMI medium at pH 6.6 or 7.4, and then, the cells were incubated for 3 h at 25 °C (a and c) or 37 °C (b and d). After discarded the medium and washed with PBS, the cells were irradiated at 1.0 J/cm². The ROS level was measured immediately after irradiation.

Folic acid inhibition. The inhibition of free Ce6 and Ce6@VNS (PC/PE–p(His)₄₀/PE–p(NIPAM)₄₀–FA = 50/25/25) uptake by folic acid was investigated in KB cells (Figure 9). KB cells were treated with 100 µL of serum-free RPMI media containing folic acid (10 µg/mL) and

incubated for 1 h at pH 6.6 and 37 °C. After discarding the medium, 100 µL of pH 6.6 serum-free RPMI media containing folic acid (10 µg/mL) and free Ce6 or Ce6@VNS (0.1 µg/mL) were administered to each cell culture and further incubated for 1 h at 37 °C. Then the intracellular Ce6 uptake (Figure 9 (A)) was measured and phototoxicity (Figure 9 (B)) was also investigated after irradiation. The presence of FA in the medium, the uptake of Ce6@VNS was disturbed to some degree, thus phototoxicity was decreased.

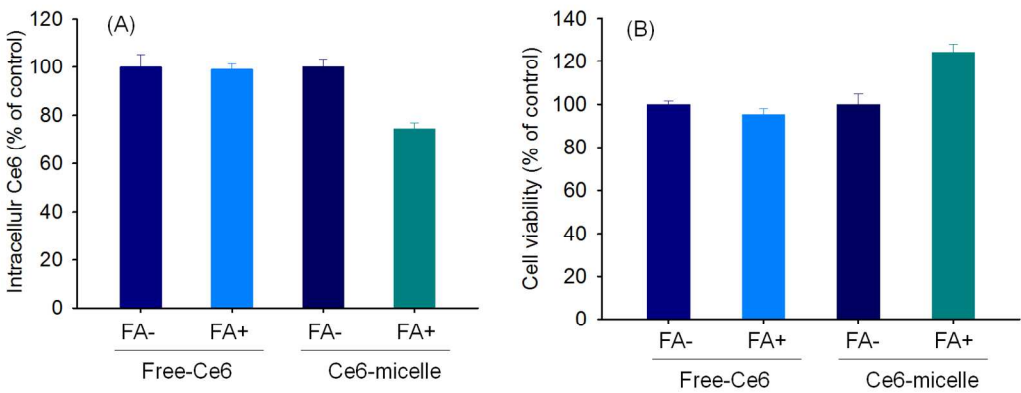


Figure 9. Inhibition of free Ce6 and Ce6@VNS (PC/PE-p(His)₄₀/PE-p(NIPAM)₄₀-FA = 50/25/25) uptake by folic acid in KB cells: (A) intracellular Ce6 level (the percentage of Ce6 relative to the control Ce6 amount without inhibitor) after the uptake of Ce6 and Ce6@VNS (0.1 µg/mL Ce6) in the presence of 10 µg/mL FA (FA+) and in the absence of FA (FA-) at pH 6.6 and 37 °C; (B) phototoxicity measured after irradiating the resulting cultures.

To evaluate apoptotic and necrotic signals in KB cells, cells were treated with free Ce6 and Ce6@VNS (0.1 µg/mL Ce6) in serum-free RPMI medium at pH 6.6 (Figure 10). The cells were incubated at 25 or 37 °C for 3 h and irradiated at 1.0 J/cm². Note that utilization of PI and FITC-conjugated Annexin V is a standard procedure to monitor the progression of apoptosis (Figure 10A), since early apoptotic cells are Annexin V-positive and PI-negative, while late apoptotic

cells are Annexin V/PI-double-positive.⁴² Treating KB cells with free Ce6 and Ce6@VNS yields no conspicuous signals indicating apoptosis, whereas necrosis signals (Figure 10B) increase markedly in KB cell populations specifically after irradiation in the Ce6@VNS treated group. The necrotic signals are more evident at higher culture temperature (37 °C) in both free Ce6 and Ce6@VNS treated groups. This phenomenon is more significant at Ce6@VNS treated group; almost twice as many necrotic cells are observed compared to the lower temperature (25 °C) culture conditions. These results are consistent with those obtained from phototoxicity tests: i.e. the highest cell death is observed when Ce6@VNS was used as a photosensitizer at pH 6.6 and 37 °C.

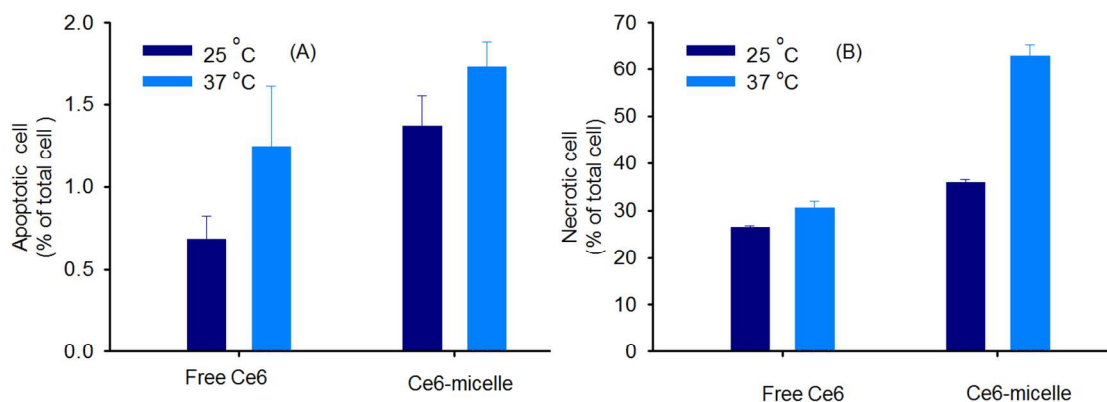


Figure 10. (A) Apoptotic and (B) necrotic signals in KB cells. The cells were incubated for 3 h in the presence of free Ce6 and Ce6@VNS (PC/PE-p(His)₄₀/PE-p(NIPAM)₄₀-FA = 50/25/25) (0.1 µg/mL Ce6) in serum-free RPMI medium at pH 6.6. After discarded the medium and washed with PBS, the cells were irradiated at 1.0 J/cm². The cells were collected and washed with PBS for flow cytometric analysis. The collected pellets were stained with PI (10 µg/mL) and FITC-annexin V (1 µg/mL) and analyzed with a FACScan flow cytometer.

CONCLUSIONS

As a means of developing stable, targetable and smart liposomes responding pH and temperature, the head groups of lecithin PE were modified by combining ROP, RAFT polymerization and click reactions. pH sensitive PE-p(His)₄₀ conjugate was synthesized by ROP of Bn-His-NCA using PE as an initiator, followed by deprotection. Temperature sensitive PE-p(NIPAM)₄₀-FA conjugate was synthesized by combining the RAFT polymerization of NIPAM by using specially designed CTA, with azide-alkyne and thiol-ene click reactions. Simple mixing of the mixture of PC, PE-p(His)₄₀ and PE-p(NIPAM)₄₀-FA yielded stable vesicles showing positive surface charge by self-assembly sized about 100 nm with narrow distribution. According to the relative ratio of PC used, the vesicular structures were modulated from vesicular sphere where the core was filled with hydrophobic p(His) units to liposome where the core was filled with aqueous solution. Accordingly Ce6 loading efficiency decreases from 45% to 19% as the relative weight ratio of PC increases from 50% to 90%. Controlled Ce6 delivery testes at different pH and temperature conditions showed that the dual-responsive nature of the Ce6@VNS (PC/PE-p(His)₄₀/PE-p(NIPAM)₄₀-FA = 50/25/25) influenced the dissociation or destabilization of the VNS, triggering Ce6 release. The FA-induced targetability and positive surface charge of the Ce6@VNS enhanced the active intake of Ce6 in the targeted tumor cells, confirming high PDT efficiency, much better than free Ce6.

■ ASSOCIATED CONTENT

Supporting Information

¹H NMR spectra of PE-p(His)₄₀ and folic acid conjugated PE-p(NIPAM)₄₀; UV-visible and fluorescence spectra of the Ce6 loaded PC/PE-p(His)₄₀/PE-p(NIPAM)₄₀-FA; DSC thermograms

of the p(NIAPM) and PC/PE-p(His)₄₀/PE-p(NIPAM)₄₀-FA. The Supporting Information is available free of charge on the ACS Publications website

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Notes

The authors declare no competing financial interest.

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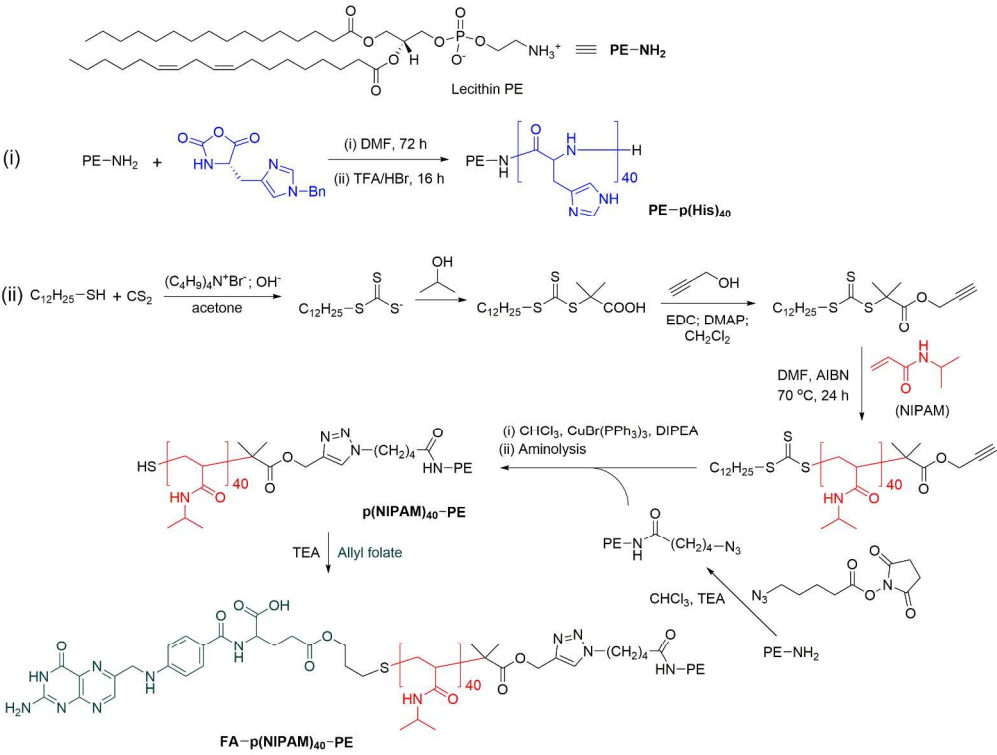
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Scheme 1. Synthesis routes of (i) PE-p(His)₄₀ and (ii) PE-p(NIPAM)₄₀-FA.
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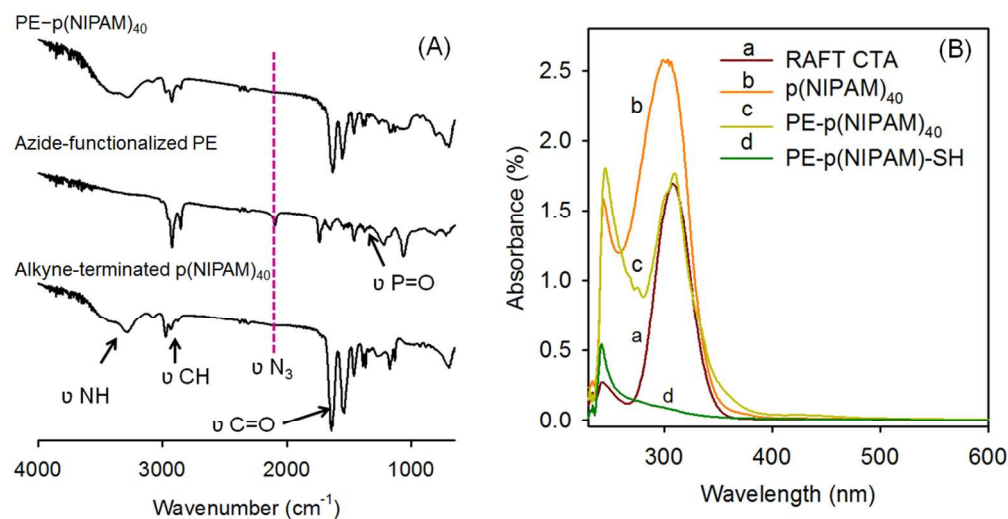


Figure 1. (A) FT-IR spectra of alkyne-terminated p(NIPAM)₄₀, azide-functionalized PE and PE-p(NIPAM)₄₀ hybrids, and (B) UV-visible spectra of RAFT CTA, p(NIPAM)₄₀, PE-p(NIPAM)₄₀ and PE-p(NIPAM)₄₀-SH.
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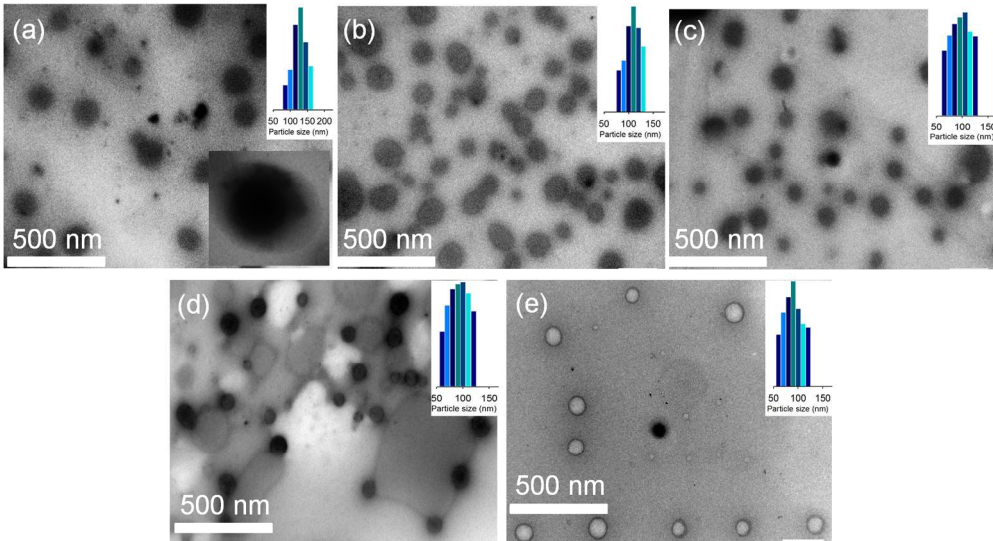


Figure 2. TEM images of nanocarriers fabricated by PC, PE-p(HIS)₄₀, and PE-p(NIPAM)₄₀-FA mixtures composed of different weight ratios: (a) 50/25/25, (b) 60/20/20, (c) 70/15/15, (d) 80/10/10 and (e) 90/5/5.
518x278mm (96 x 96 DPI)

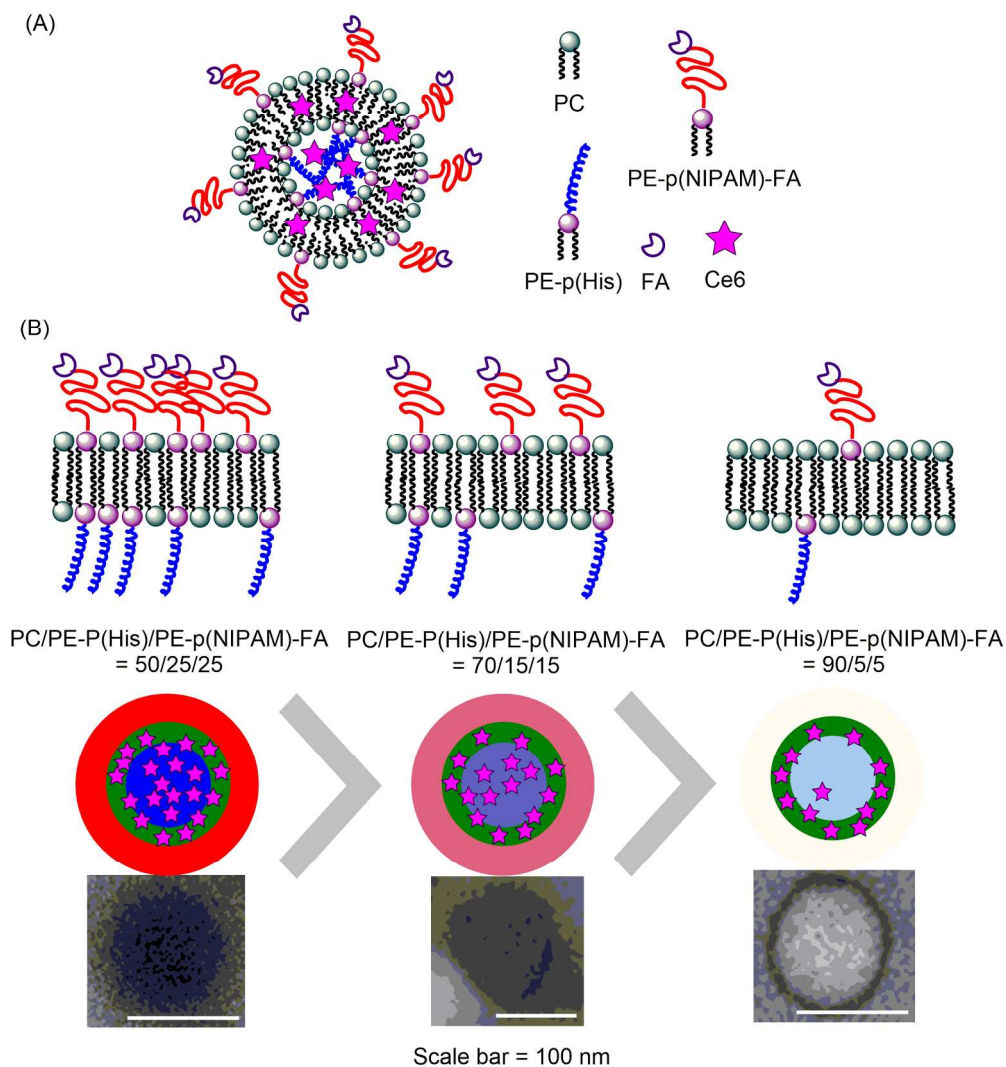


Figure 3. (A) Diagrammatic representation of VNS bearing Ce6 fabricated by PC, PE-p(His)₄₀, and PE-p(NIPAM)₄₀-FA mixtures; (B) morphological transition of VNS from semisolid VNSs to liposomes by modulating relative composition of PC, PE-p(His)₄₀, and PE-p(NIPAM)₄₀-FA.

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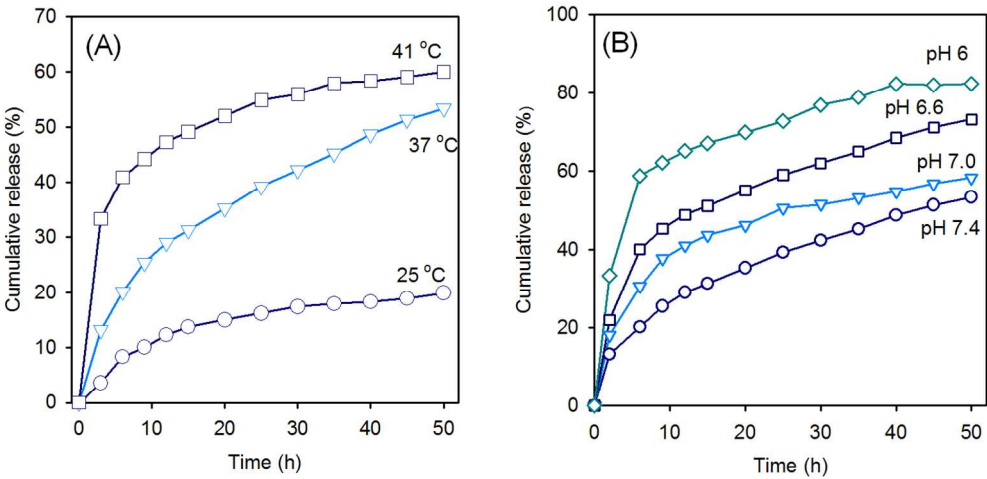


Figure 4. Time-dependent releases of Ce6 from the VNS fabricated by PC/PE-p(His)₄₀/PE-p(NIPAM)₄₀-FA (50/25/25) estimated (A) at different temperatures (pH = 7.4) and (B) at different pH values (temperature = 37 °C).
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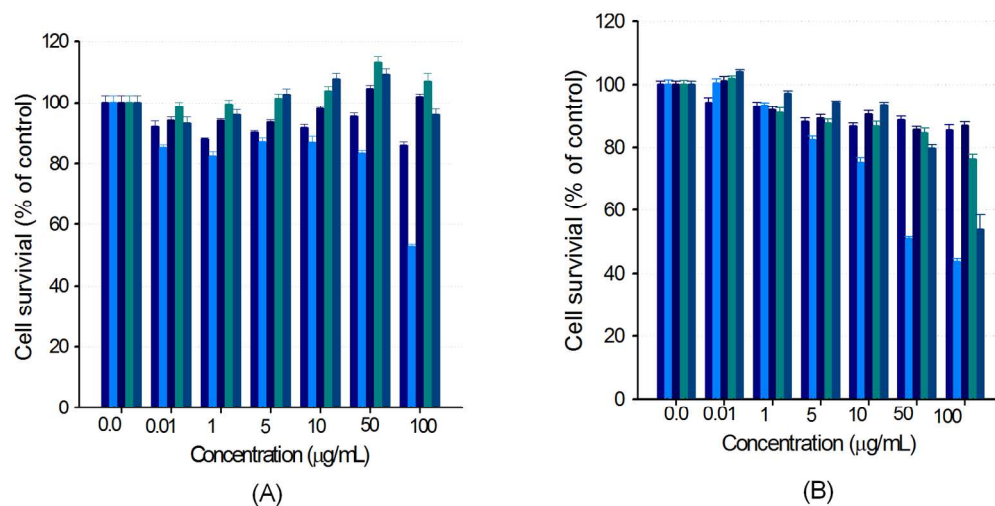


Figure 5. Intrinsic cytotoxicity of VNSs fabricated by PC/PE-p(His)₄₀/PE-p(NIPAM)₄₀-FA mixture in different formulations against (A) CCD986-Sk and (B) KB cells. After starvation for 18 h, the cells were treated with various concentration of polymer micelle. Cell viability was revealed as percent of control. In each concentration PC/PE-p(His)₄₀/PE-p(NIPAM)₄₀-FA ratio is corresponding to 50/25/25, 60/20/20, 70/15/15, 80/10/10 and 90/5/5 in order from the left.

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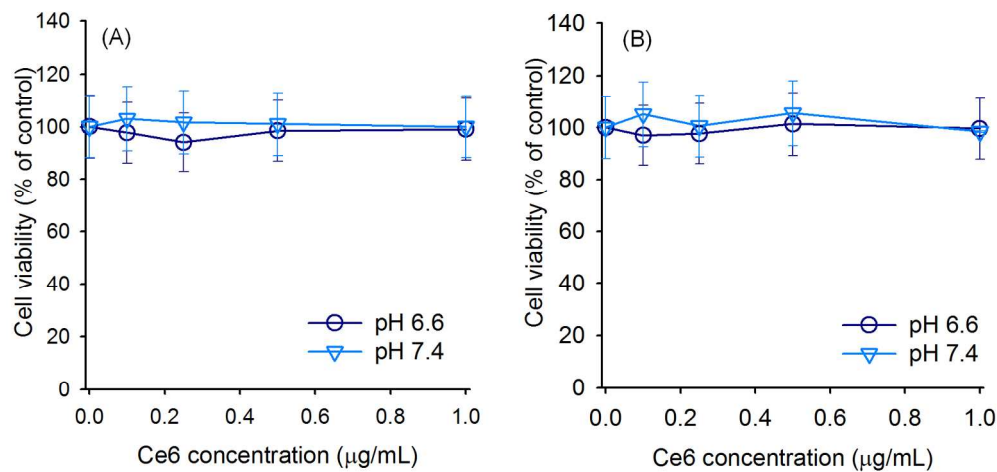


Figure 6. Dark toxicity of (A) free Ce6 and (B) Ce6@VNS (PC/PE-p(His)₄₀/PE-p(NIPAM)₄₀-FA = 50/25/25) against KB cells; KB cells were incubated with 0-1 μg/mL Ce6 or Ce6@VNS in the dark at different pH conditions for 3 h. Cell survival was expressed as the percentage of the control (not exposed to Ce6 or Ce6@VNS) and was determined using the MTT assay.
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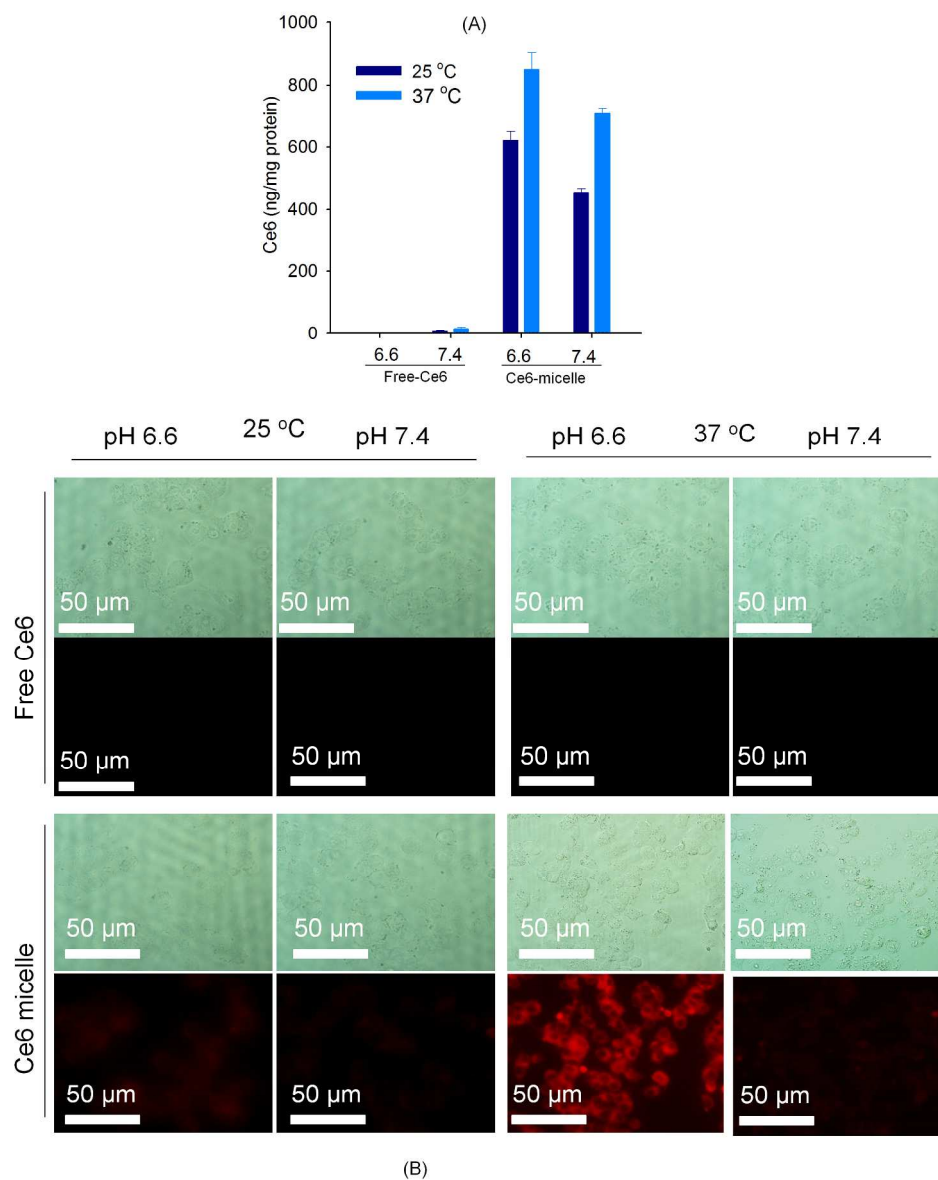


Figure 7. Intracellular accumulation of Ce6 in KB cells at different pH and temperature conditions: (A) comparison of the amount of intracellular Ce6 uptake of free Ce6 with Ce6@VNS (PC/PE-p(His)₄₀/PE-p(NIPAM)₄₀-FA = 50/25/25) and (B) optical and confocal laser scanning microscopic images of KB cells. The KB cells were incubated with 0.1 μg/mL free Ce6 or Ce6@VNS for 3 h. The amount of intracellular Ce6 was determined fluorometrically.

717x905mm (96 x 96 DPI)

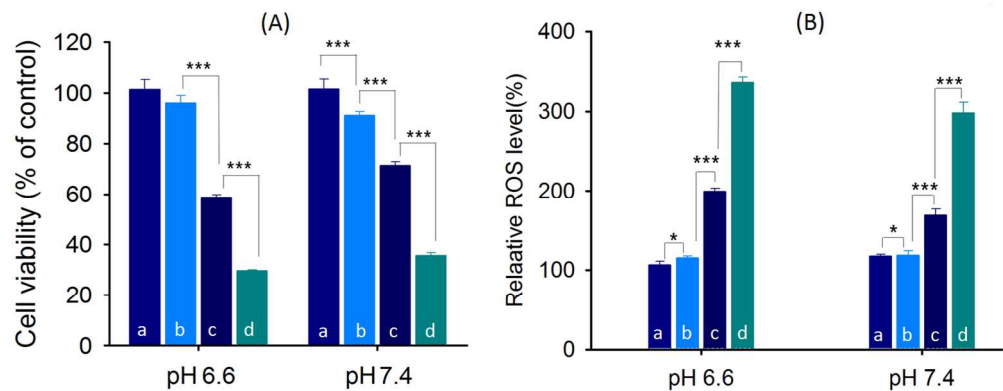


Figure 8. (A) Phototoxicity of free Ce6 (a and b) and Ce6@VNS (PC/PE-p(His)₄₀/PE-p(NIPAM)₄₀-FA = 50/25/25) (c and d) against KB cells irradiated with 1.0 J/cm² light after incubating them with 0.1 µg/mL Ce6 or Ce6@VNS at pH 6.6 or 7.4 and at 25 °C (a and c) or 37 °C (b and d) for 3 h; (B) relative ROS level after photodynamic treatments in the presence of free Ce6 (a and b) and Ce6@VNS (c and d). For the measurement of ROS level KB cells were treated with free Ce6 and Ce6@VNS with DCFH-DA at a final concentration of 20 µM in phenol red serum-free RPMI medium at pH 6.6 or 7.4, and then, the cells were incubated for 3 h at 25 °C (a and c) or 37 °C (b and d). After discarded the medium and washed with PBS, the cells were irradiated at 1.0 J/cm². The ROS level was measured immediately after irradiation.

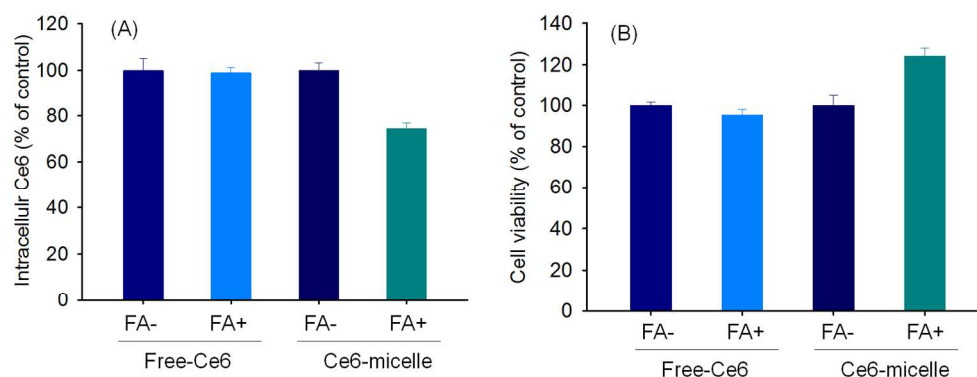


Figure 9. Inhibition of free Ce6 and Ce6@VNS (PC/PE-p(His)₄₀/PE-p(NIPAM)₄₀-FA = 50/25/25) uptake by folic acid in KB cells: (A) intracellular Ce6 level (the percentage of Ce6 relative to the control Ce6 amount without inhibitor) after the uptake of Ce6 and Ce6@VNS (0.1 $\mu\text{g/mL}$ Ce6) in the presence of 10 $\mu\text{g/mL}$ FA (FA+) and in the absence of FA (FA-) at pH 6.6 and 37 $^{\circ}\text{C}$; (B) phototoxicity measured after irradiating the resulting cultures.
468x196mm (96 x 96 DPI)

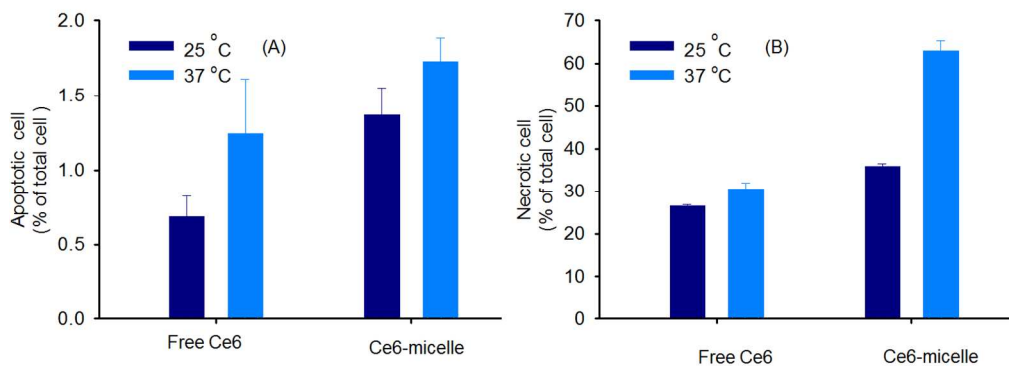
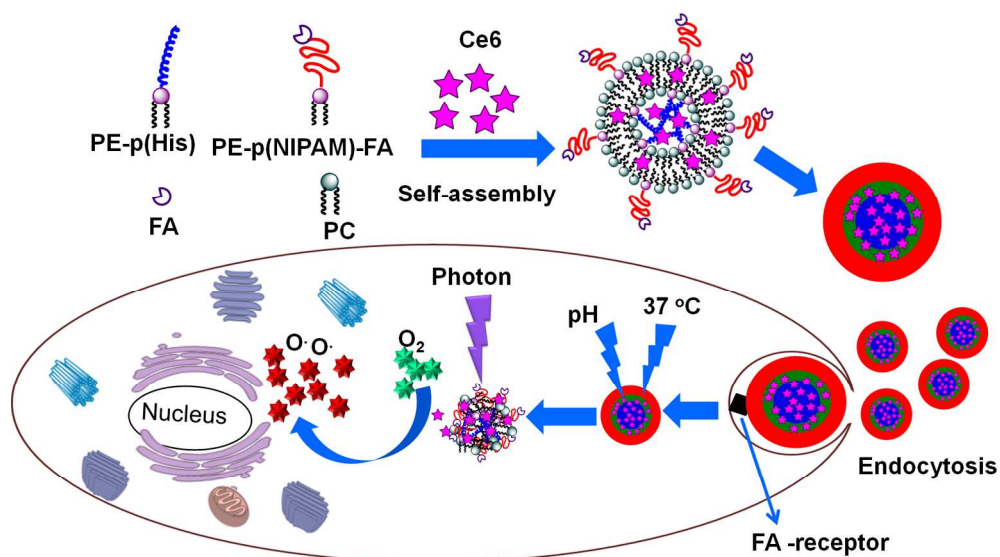


Figure 10. (A) Apoptotic and (B) necrotic signals in KB cells. The cells were incubated for 3 h in the presence of free Ce6 and Ce6@VNS (PC/PE-p(His)₄₀/PE-p(NIPAM)₄₀-FA = 50/25/25) (0.1 μ g/mL Ce6) in serum-free RPMI medium at pH 6.6. After discarded the medium and washed with PBS, the cells were irradiated at 1.0 J/cm². The cells were collected and washed with PBS for flow cytometric analysis. The collected pellets were stained with PI (10 μ g/mL) and FITC-annexin V (1 μ g/mL) and analyzed with a FACScan flow cytometer. 396x152mm (96 x 96 DPI)



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482x270mm (96 x 96 DPI)