Electronic Supplementary Material (ESI) for Biomaterials Science.

Dynamic-Responsive Virus-Mimetic Nanocapsules Facilitate Protein Drug Penetration and Extracellular-Specific Unpacking for Antitumor Treatment

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1. Experimental Section

1.1 Materials

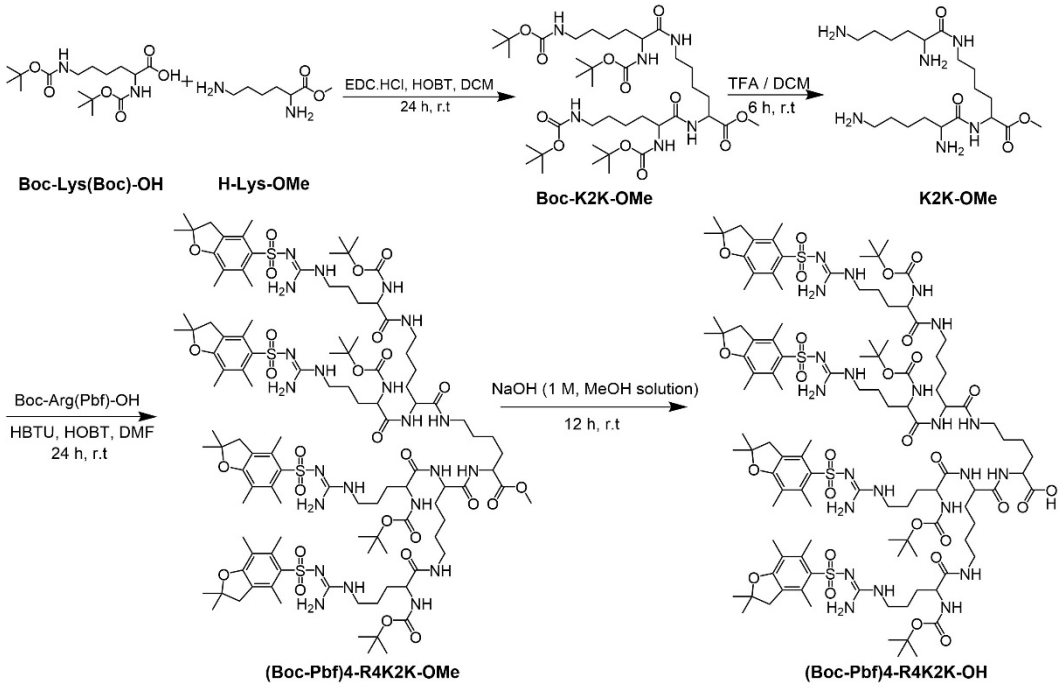
Boc-Lys(Boc)-OH, Boc-Arg(Pbf)-OH, H-Lys-OMe, N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC.HCl), O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluorophosphate (HBTU), PyBOP, and 1-Hydroxybenzotriazole hydrate (HOBT) were purchased from GL Biochem (Shanghai) Ltd. Trifluoroacetic acid (TFA), N-Ethyldiisopropylamine (DIPEA), fluorescein-5-isothiocyanate (FITC) and Cyanine 5 (Cy5) mono-reactive NHS esters were purchased from Aladdin Chem. Co. Ltd. TRAIL was purchased from Sino Biological. Organic solvents, including dichloromethane (DCM), N,N-Dimethylformamide (DMF), DMSO, MeOH, and petroleum ether, were purchased from Chron Chemicals. NaCl and $NaHCO_3$ were obtained from Sinopharm Chemical reagent Co., Ltd. Dulbecco's modified eagle medium (DMEM) high glucose culture medium, fetal bovine serum (FBS), and penicillin-streptomycin solution (1000x) were purchased from ThermoFisher Scientific. Cell counting kit-8 (CCK-8 kit, Dojindo Laboratories, Japan), Hoechst 33342 (Sigma-Aldrich, USA), and propidium iodide (PI, Sigma-Aldrich, USA) were obtained for biological research.

Dulbecco's modified eagle medium (DMEM) high glucose culture medium, fetal bovine serum (FBS) and penicillin-streptomycin solution (1000x) were purchased from ThermoFisher Scientific. Lovo/R cells were cultured in DMEM high glucose culture medium containing 10% FBS and 1% penicillin-streptomycin at 37 ℃ in a humidified atmosphere containing 5% CO_2.

Mass spectrum assay was conducted on matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-ToF MS, Bruker, UltrafleXtreme, USA). H nuclear magnetic resonance spectrum was obtained by an NMR spectrometer (Bruker, AVANCE III HD 400 MHz, Germany). Nanostructure was imaged by a transmission electron microscope (TEM, JEM-2100Plus, Japan). The secondary structure of TRAIL and nanoparticles were measured by a circular binary chromatograph (Bio-Logic, France). The fluorescence curves were measured by a fluorescence spectrophotometer (Hitachi, F-7000, Japan). The cells were imaged by a confocal laser scanning microscopy (CLSM, Olympus, FV1200, Japan). In vivo drug distribution investigation was conducted with an in vivo imaging system (Czliper, Lumina xr, USA).

1.2 Synthesis of Peptide Dendrimer

Synthesis of (Boc-Pbf)4-R4K2K-OH

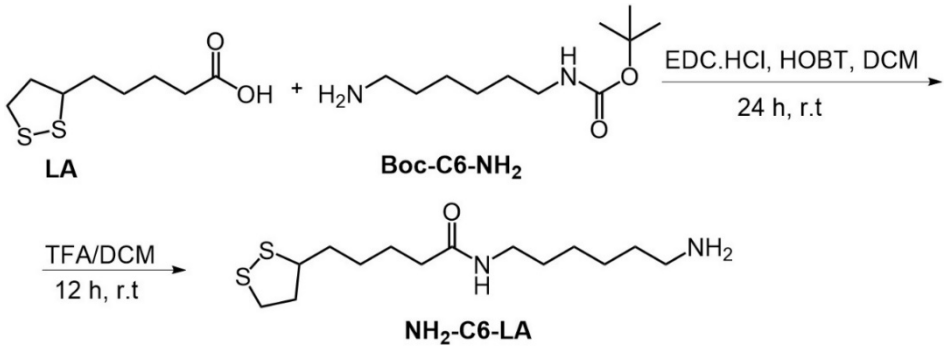


Scheme S1. Synthesis of (Boc-Pbf)4-R4K2K-OH.

Boc-Lys(Boc)-OH (7.13g, 20.59 mmol), H-Lys-OMe (2.00 g, 8.58 mmol), EDC.HCl (3.95 g, 20.59 mmol) and HOBT (2.78 g, 20.59 mmol) were dissolved in DCM under N_2 atmosphere. DIPEA (9.0 mL, 51.48 mmol) was added to the mixture and kept in the ice bath for 30 min. The mixture was stirred for 24 h. Excess DCM was added to the reaction. The mixture was washed with saturated NaCl solution, $NaHCO_3$ solution and HCl solution (1 M) for 3 times. Then the solution was dried with anhydrous sodium sulfate overnight. The solution was concentrated with reduced pressure. And the residues were purified by a silica column with petroleum ether: ethyl acetate of 1:2. The Boc-K2K-OMe was dissolved in DCM and treated with TFA (Boc group: TFA = 1:10, mol/mol) for 12 h. After removing the solvent, H-K2K-OMe was precipitated by diethyl ether.

K2K-OMe (1.50 g, 3.60 mmol), Boc-Arg(Pbf)-OH (9.11 g, 17.30 mmol), HBTU (6.56 g, 17.30 mmol), and HOBT (2.14 g, 15.85 mmol) were dissolved in DMF under N_2 atmosphere. DIPEA (10.0 mL, 57.65 mmol) was added to the mixture and kept in ice bath for 30 min. The mixture was stirred for 48 h. DMF was removed by a rotary evaporator with reduced pressure. Excess DCM was added to the residue. And the mixture was washed as above 3 times. Then the solution was dried with anhydrous sodium sulfate overnight. The solution was concentrated with reduced pressure. And the residues were purified by a silica column with DCM and methanol with a ratio of 15:1 to obtain (Boc-Pbf)4-R4K2K-OMe. (Boc-Pbf)4-R4K2K-OMe was treated with NaOH methanol solution (1 M, methyl groups: NaOH = 1:10, mol/mol) for 12 h. The MeOH was removed to obtain (Boc-Pbf)4-R4K2K-OH.

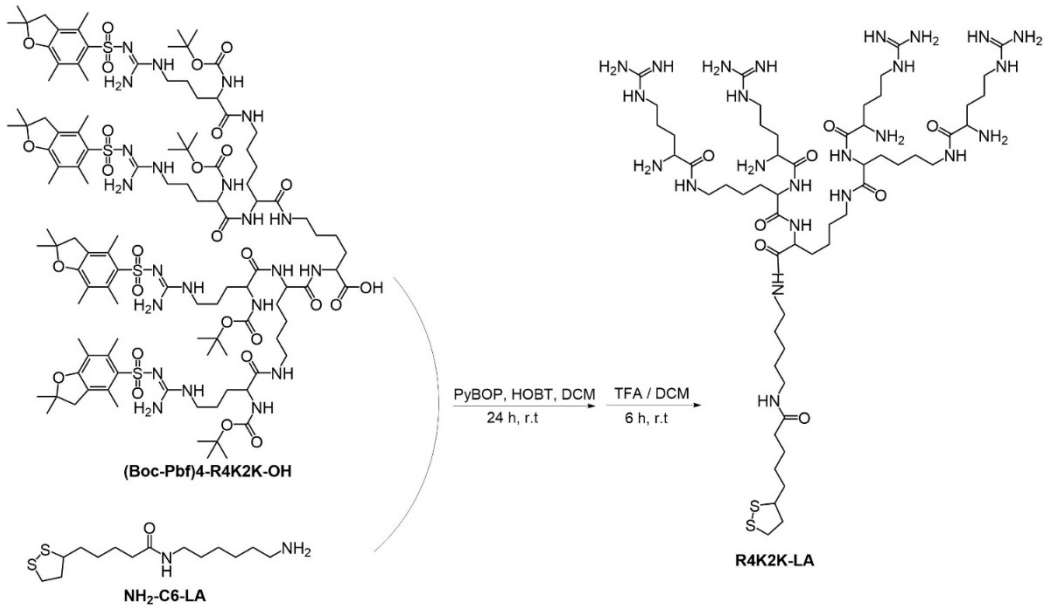
Synthesis of NH_2-C6-LA



Scheme S2. Synthesis of NH_2-C6-LA.

N-Boc-1,6-hexanediamine (1.23 g, 4.85 mmol), (alpha-)lipoic acid (1.00 g, 4.85 mmol), EDC.HCl (1.11 g, 5.82 mmol) and HOBt (0.79 g, 5.82 mmol) were dissolved in DCM under N_2 atmosphere. DIPEA (5.1 mL, 29.08 mmol) was added to the mixture and kept in ice bath for 30 min. The mixture was stirred for 24 h. Excess DCM was added to the residue. And the mixture was washed as above for 3 times. Then the solution was dried with anhydrous sodium sulfate overnight. The solution was concentrated with reduced pressure. And the residues were purified by a silica column with DCM and ethyl acetate with a ratio of 1:2 to obtain Boc-C6-LA. The Boc-C6-LA was treated with TFA (Boc group: TFA = 1:10, mol/mol) for 12 h. After removing the solvent, NH_2-C6-LA was precipitated by diethyl ether.

Synthesis of R4K2K-LA



Scheme S3. Synthesis of R4K2K-LA.

(Boc-Pbf)4-R4K2K-OH (1.00 g, 0.41 mmol), N_H2-C6-LA (0.15 g, 0.49 mmol), PyBOP (0.43 g, 0.82 mmol) and HOBT (0.07 g, 0.49 mmol) were dissolved in DMF under N_2 atmosphere. DIPEA (0.4 mL, 2.46

mmol) was added to the mixture and kept in ice bath for 30 min. The mixture was stirred for 48 h. DMF was removed by rotary evaporator with reduced pressure. The residue was dissolved by DCM and washed as above. The solution was dried with anhydrous sodium sulfate overnight and concentrated with reduced pressure. The residues were purified by a silica column with DCM and methanol with a ratio of 15:1 to obtain (Boc-Pbf)4-R4K2K-LA. (Boc-Pbf)4-R4K2K-LA was treated with TFA (Boc group: TFA = 1:10, mol/mol) for 18 h. TFA and DCM was removed. And diethyl ether was added to the residue to obtain yellow solid R4K2K-LA.

Synthesis and stabilization of DR4K2K-LA

DMA and R4K2K-LA was dissolved in PBS buffer and reacted for 48 h. NaOH (1 M) solution was used to maintain the pH at a relative stable value of ~ pH 8. The DR4K2K-LA was purified by dialysis (MWCO 1000) in water and freeze dried. Then the DR4K2K-LA (100 mg) was dissolved in 20 mL water. DTT (1% equiv of disulfide bond) was added to the solution and kept the pH at ~8. The solution was purified by dialysis (MWCO 1000) in water and freeze dried to obtain ACs.

Preparation of P-VMNs

TRAIL was incubated with ACs solution (200 μg mL^{-1}) at room temperature for 15 min to obtain P-VMNs. Then the solution was added to RGD-CSO solution (50 μg mL^{-1}). The size and zeta potential of P-VMNs was measured by DLS. The ratio of RGD-CSO and ACs in P-VMNs for *in vitro* and *in vivo* investigation was determined by size and zeta potential.

To investigate the interaction between TRAIL and ACs, TRAIL and FITC (2% of TRAIL, mol/mol) were stirred at 4℃ for 24 h. Then the solution was purified by ultracentrifuge to obtain FITC-labeled TRAIL. ACs were reacted with BHQ-1 to obtain BHQ-labeled ACs. FITC-labeled TRAIL was incubated with different concentrations of BHQ-labeled ACs from 0 – 40 μg mL^{-1}. And the solution was measured by a fluorescence spectrophotometer.

1.3 Transmission electron microscopy (TEM)

P-VMNs were dissolved in water to prepare 300 μg mL^{-1} solution. Drop the solution on a copper grid and adsorbed for 5 min. The residual solution was adsorbed by filter paper. Then the copper grid was dried at room temperature. The nanoparticles were imaged by TEM.

1.4 Circular Dichroism (CD) analysis

P-VMNs were dissolved in water and measured by a dichroism spectropolarimeter. CD spectra were the average of three scans obtained by collecting data from 250 to 190 nm.

P-VMNs were incubated at pH 6.5. The TRAIL released from P-VMNs was collected by ultrafiltration. And the released TRAIL in ultrafiltrate was measured by dichroism spectropolarimeter.

1.5 In vitro TRAIL release

P-VMNs (20 μg mL^{-1} TRAIL) was incubated in pH 6.5 and pH 7.4 PBS buffer for different times. The released TRAIL was collected by ultrafiltration with an ultrafilter tube of MWCO 50kD. Then the concentration of TRAIL was measured by BCA protein assay kit.

1.6 Protein adsorption assay

Arginine-dendrons (R-Den), ACs, P-ACs, and P-VMNs (300 μg mL^{-1}) were incubated with BSA or FBS (200 μg mL^{-1}) at 37℃ with mild shake. At given times, the samples were centrifuged at 8000g for 15 min. The unabsorbed protein in the supernatant was quantified by Nanodrop at 280 nm. The concentration of BSA and FBS was calculated by a standard calibration curve.

1.7 Cell membrane binding of TRAIL

P-VMNs (10 μg mL^{-1} of TRAIL) were incubated with LoVo/R cells at pH 7.4 and pH 6.5 conditions for 1 h. Then the cells were washed with PBS buffer 2 times. Cell membrane was stained with DiD dye (1 μg mL^{-1}) for 20 min. The cells were washed with PBS and imaged by CLSM.

1.8 Cell viability assay

LoVo/R cells were seeded on 96-well plate with 8000 cells per well and cultured overnight. Then the cells were incubated with TRAIL and P-VMNs at pH 7.4 and pH 6.5 for 24 h. Then the media was removed and added fresh culture media containing 10% CCK8. The cells were incubated with CCK8 for 1.5 h at 37℃. The OD value at 450 nm was measured. The cell viability was calculated with following formula.

Cell viability=$(OD_{sample}-OD_{blank}) /(OD_{control}-OD_{blank})\times 100\%$

1.9 Live/dead cell staining

LoVo/R cells (5000 cells per dish) were seeded on confocal dishes and cultured for 24 h. The cells were incubated with TRAIL and P-VMNs (100 ng mL^{-1} TRAIL) at pH 7.4 and pH 6.5 for 24 h. Then the cells were stained with FDA (10 μg mL^{-1}) and PI (1 μg mL^{-1}) for 15 min and imaged by an inverted fluorescence microscope.

1.10 In vitro penetration investigation

LoVo/R cells (2000 cells per well) were seeded on 1% agarose-coated 6-well plate and culture in 37 °C humidified incubator with 5% CO_2 to form multicellular tumor spheroids (MTSs). The culture media was replaced with fresh culture media every three day. When the MTSs with a diameter of ~200 μm, MTSs were

transferred to confocal dishes and incubated with TRAIL and P-VMNs in pH 7.4 and pH 6.5 conditions (FITC-labeled TRAIL: 10 μg mL^{-1}) for 6 h. Then the MTSs were washed with PBS two times and scanned by CLSM. The 3D surface plot images were analyzed by ImageJ software.

1.11 In vivo tumor penetration investigation

Animals and tumor xenograft models

All animal experiments were approved by the ethics committee Hunan University with the approval number of SYXK(XIANG)2018-0006. 1×10^6 LoVo/R cells were subcutaneously injected onto the right flank of BALB/c mice. The mice were used for *in vivo* study until the tumor volume reached ~100 mm^3.

In vivo tumor penetration investigation

Cy5-labeled TRAIL was used for in vivo investigation. LoVo tumor-bearing BALB/c mice was intravenous injected with Cy5-labeled P-VMNs(-T) and P-VMNs (Cy5-labeled TRAIL: 10 μg kg^{-1}). After 12 h post-injection, the mice were sacrificed. And tumor tissues were sliced and stained with CD31 antibody for tumor vessel and DAPI for cell nucleus. These slices were observed by CLSM.

1.12 In vivo antitumor investigation

LoVo/R bearing mice were intravenously injected with saline, DOX.HCl, P-VMNs and P-VMNs(-T) (TRAIL: 10 μg kg^{-1}) every three days for 4 times. The tumor volume was recorded by caliper every three days until 24 days. The tumor volume was calculated by the following formula: $V=(L\times W^2)/2$, where L was the longer diameter of tumor and W for the shorter one. At the end, tumors were excised for pathologic analysis.

1.13 HUVEC tube formation assay

The μ-slide plate was used for tube formation assay. 10 μL Matrigel was added to the lower chamber and incubated in 37℃ for 20 min. Then stable GFP-expressing HUVEC cells were pretreated with P-VMNs and P-VMNs(-T) for 12 h (50 ng mL^{-1} TRAIL). Then the cells (3000 cells in 50 μL culture media) were seeded in the upper chamber and cultured for 8 h. The cells were imaged by inverted fluorescence microscope.

2. Results

2.1 Characterizations

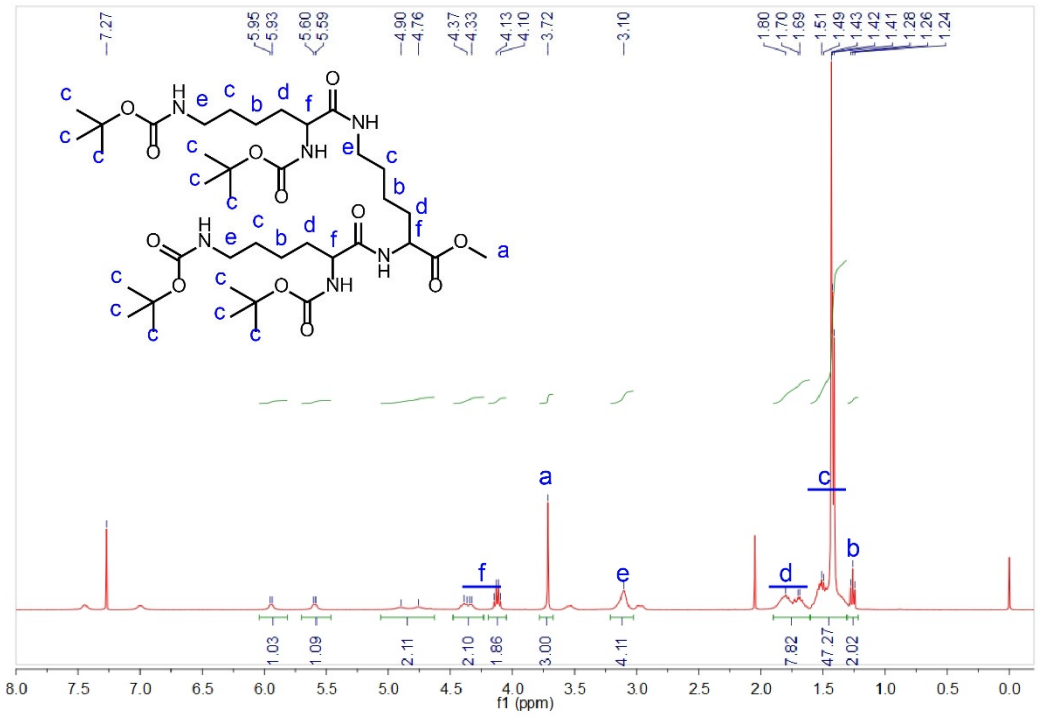


Fig. S1. ^{1}H NMR spectrum of Boc-K2K-OMe in $CDCl_3$ (400 mHz).

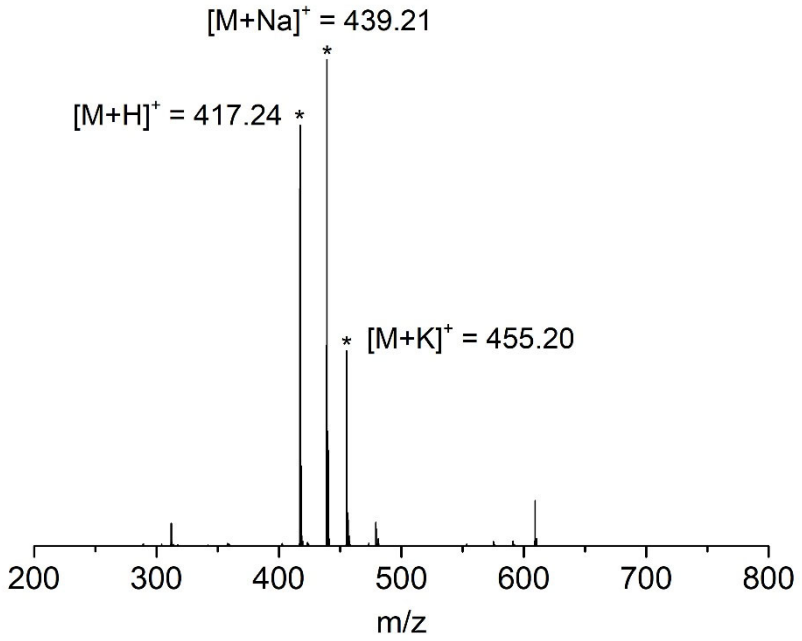


Fig. S2. ESI mass spectrum of H-K2K-OMe (m/z, $[M+H]^+$=417.31 (calculated), $[M+H]^+$=417.24 (observed)).

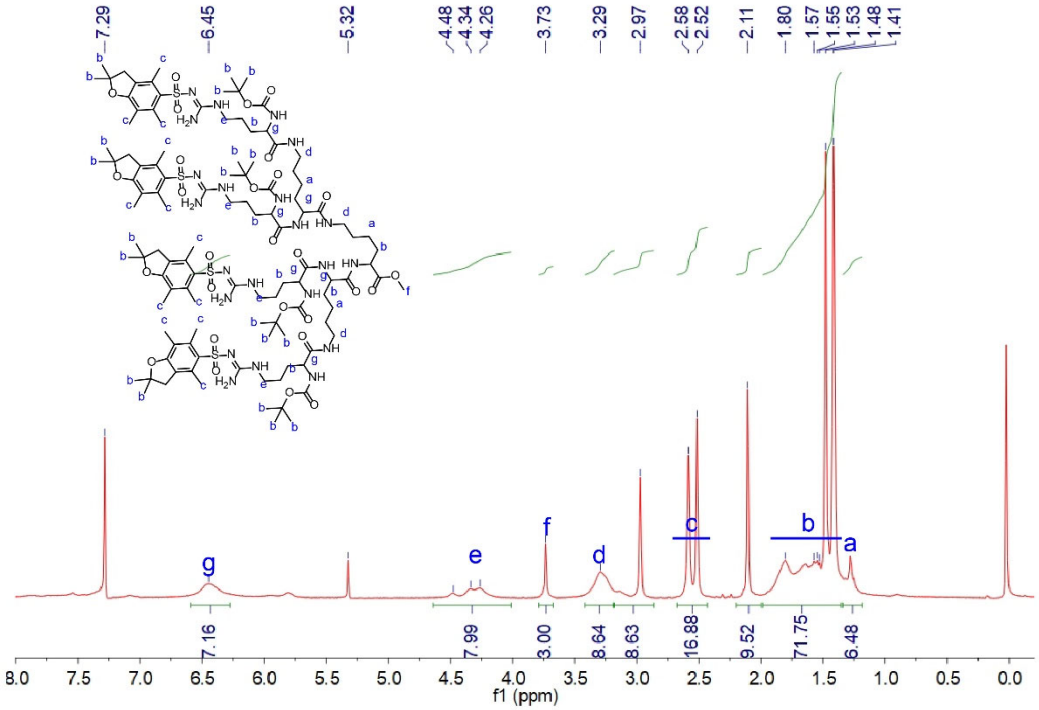


Fig. S3. ^{1}H NMR spectrum of (Boc-Pbf)4-R4K2K-OMe in $CDCl_3$ (400 mHz).

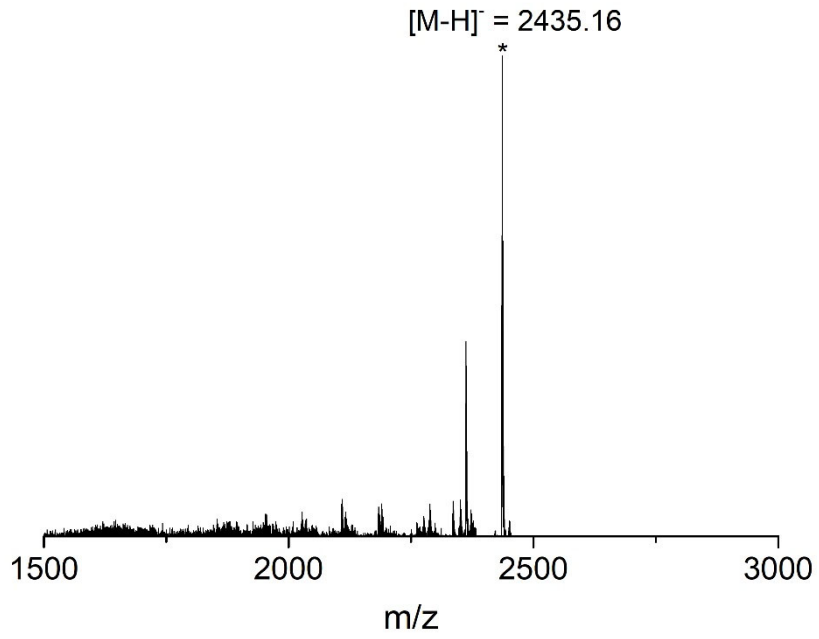


Fig. S4. ESI mass spectrum of (Boc-Pbf)4-R4K2K-OH (m/z, $[M-H]^-$=2435.24 (calculated), $[M-H]^-$ =2435.16 (observed)).

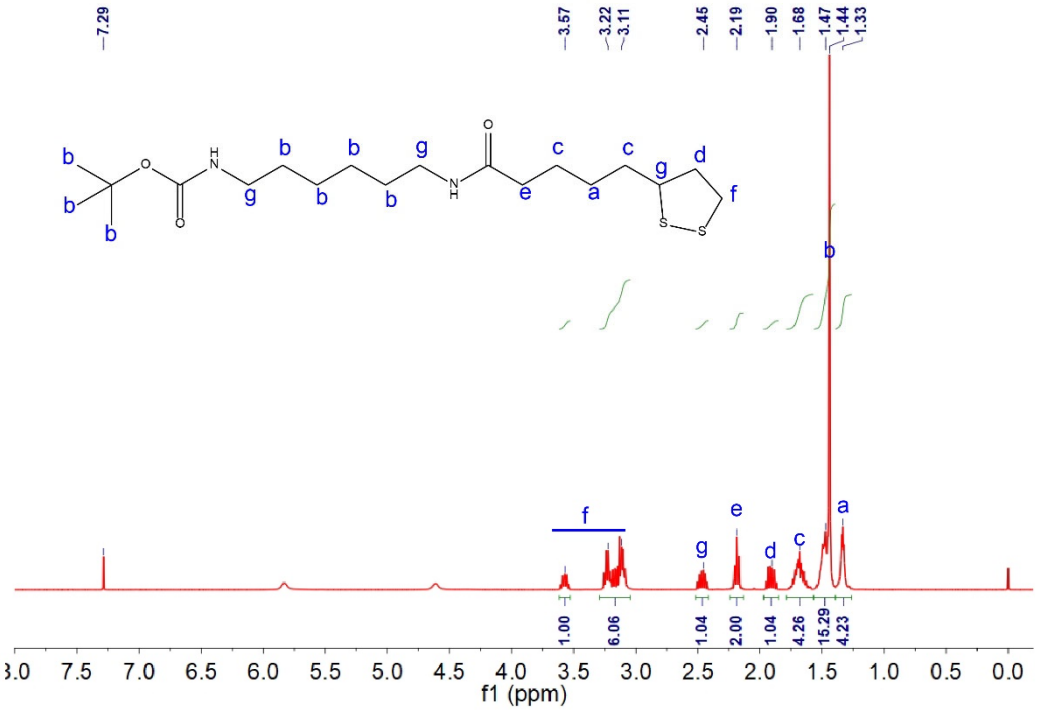


Fig. S5. ^{1}H NMR spectrum of Boc-C6-LA in $CDCl_3$ (400 mHz).

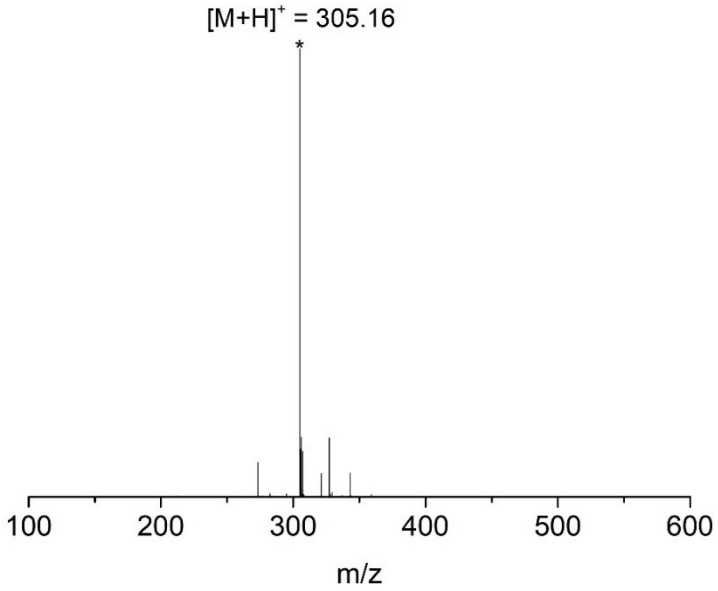


Fig. S6. ESI mass spectrum of NH_2-C6-LA (m/z, $[M+H]^+$=305.16 (calculated), $[M+H]^+$= 305.16(observed)).

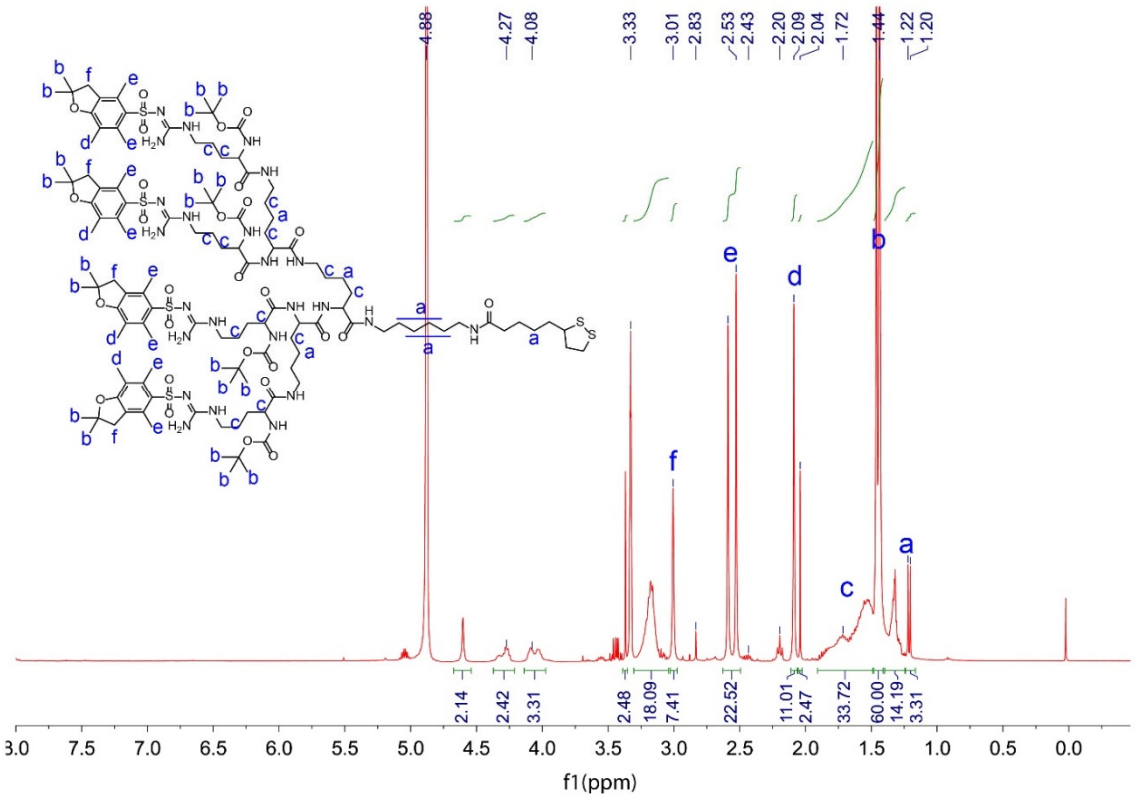


Fig. S7. ^{1}H NMR spectrum of (Boc-Pbf)4-R4K2K-LA in CD_3OD (400 mHz).

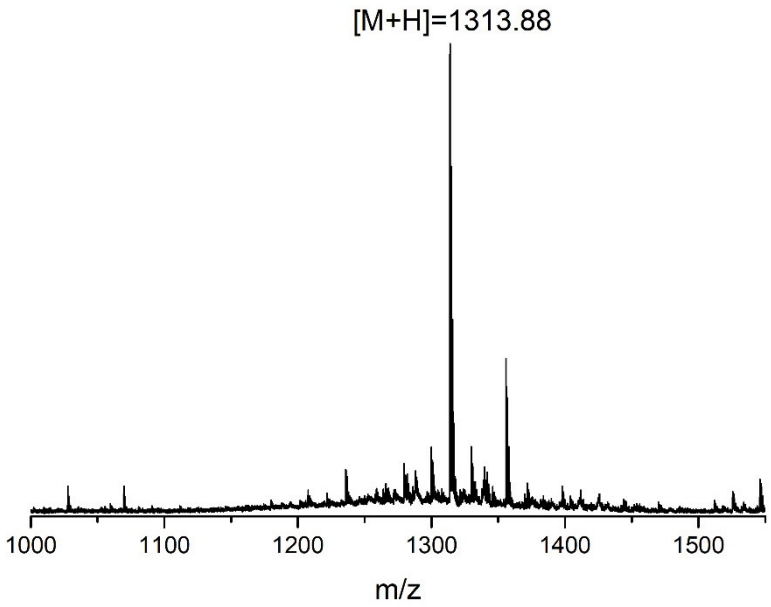


Fig. S8. MALDI-TOF mass spectrum of R4K2K-LA (m/z, $[M+H]^+$=1313.85 (calculated), $[M+H]^+$= 1313.88 (observed)).

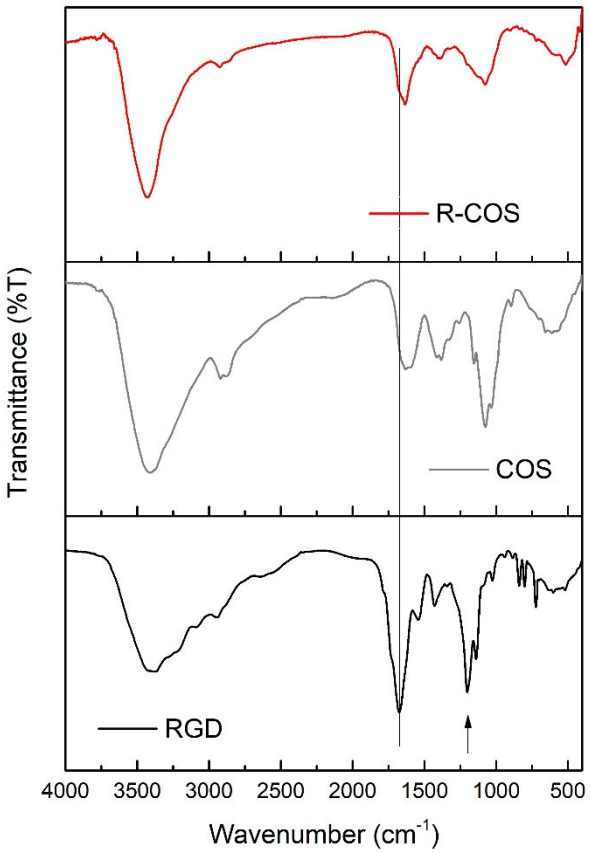


Fig. S9. FTIR Spectrum of RGD-COS.

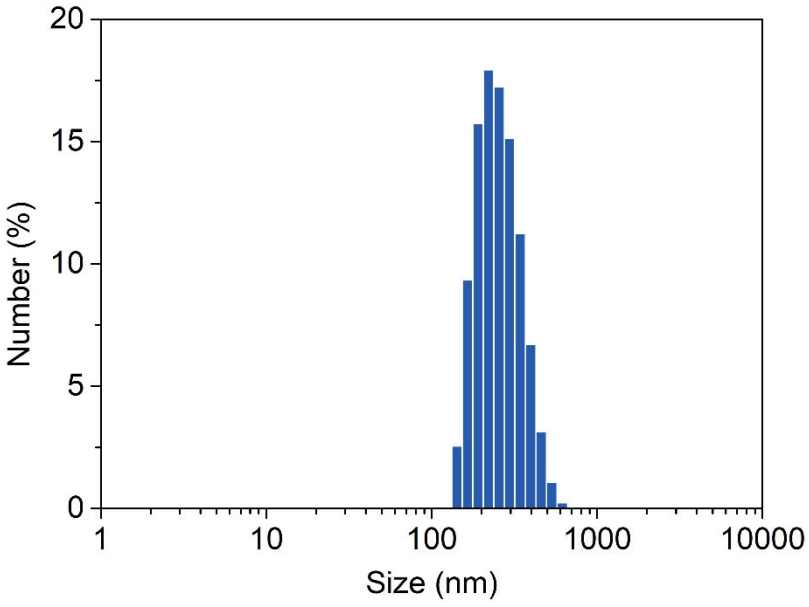


Fig. S10. Size of nano-assemblies of amphiphilic dendritic peptides.

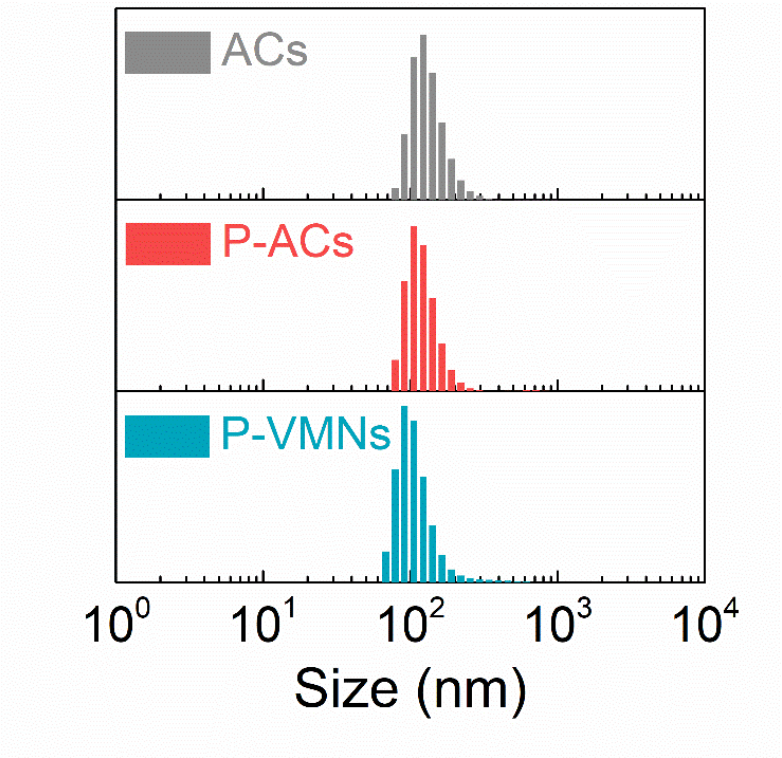


Fig. S11. Size of ACs, P-ACs and P-VMNs.

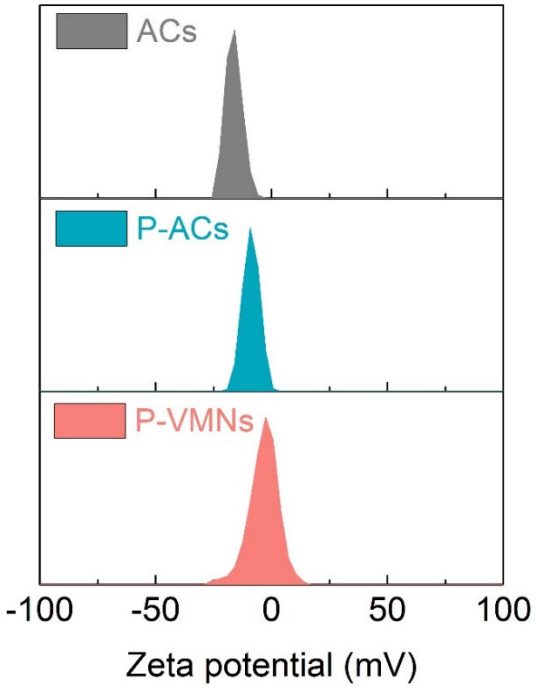


Fig. S12. Zeta potential of ACs, P-ACs and P-VMNs.

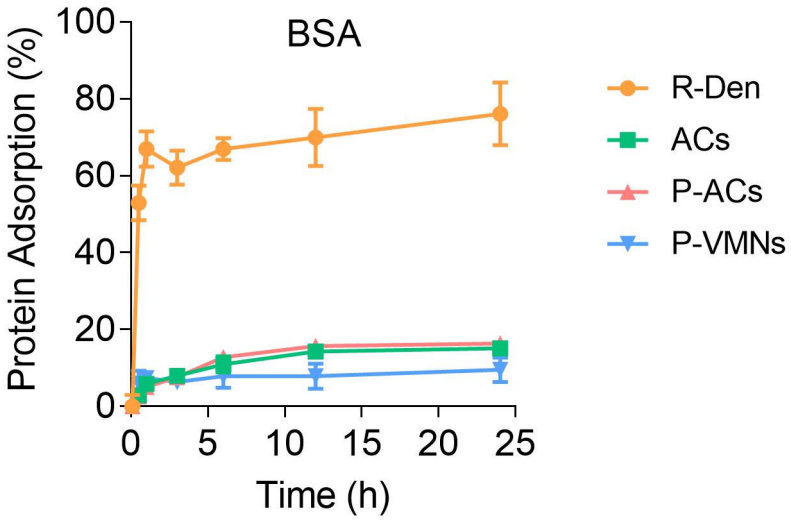


Fig. S13. BSA adsorption of R-Den, ACs, P-ACs and P-VMNs.

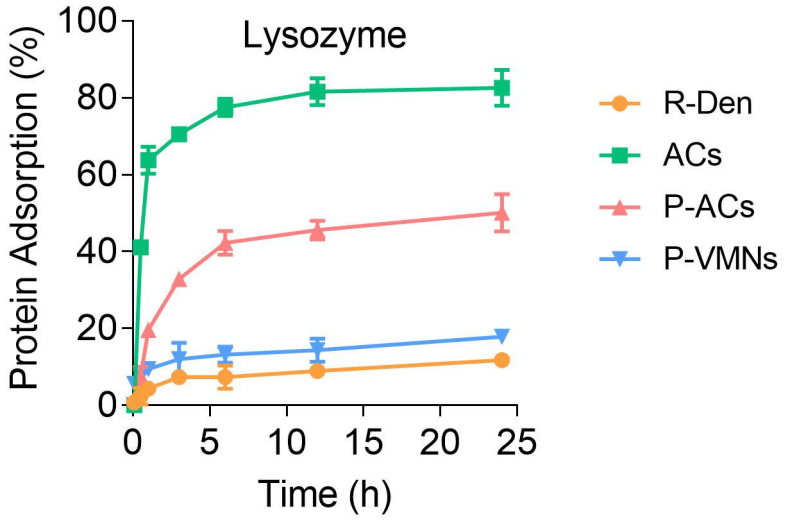


Fig. S14. Lysozyme adsorption of R-Den, ACs, P-ACs and P-VMNs.

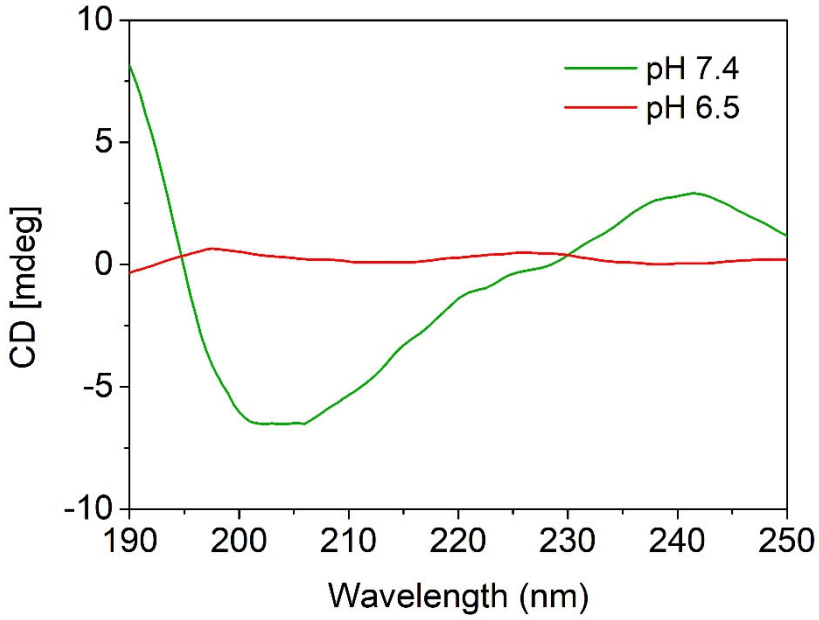


Fig. S15. CD spectrum of P-VMNs at pH 7.4 and pH 6.5.

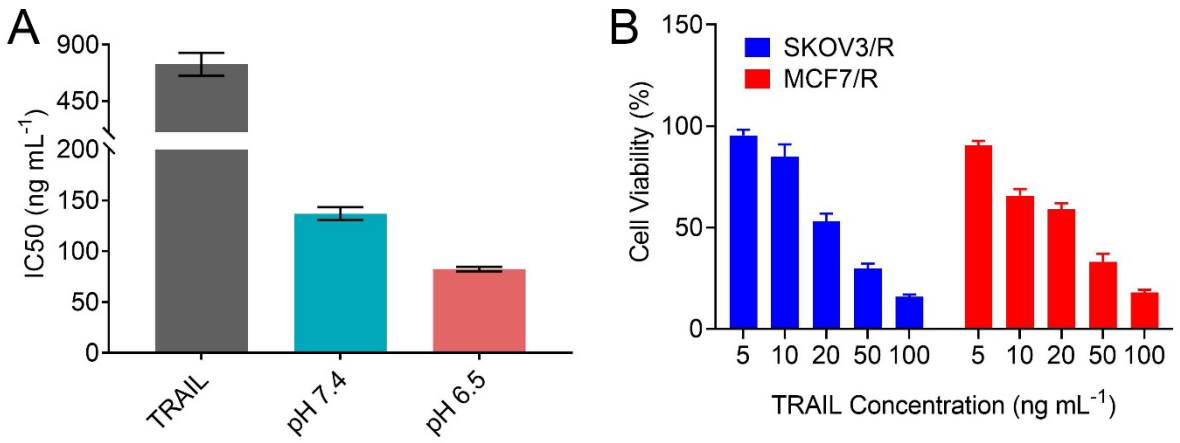


Fig. S16. A. IC50 of TRAIL and P-VMNs at pH 7.4 and pH 6.5 condition. B. Cell viability of SKOV3/R and MCF7/R cells treatment with free P-VMNs at pH 6.5 condition (n = 6, mean ± SD).

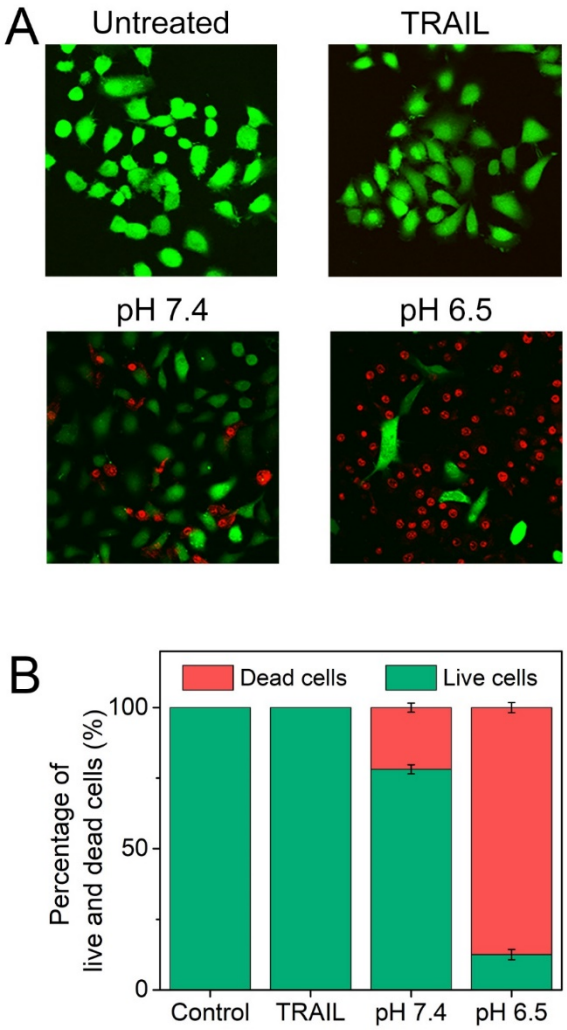


Fig. S17. (A) Fluorescence images and (B) percentage of live and dead cells after LoVo/R cells treatment with TRAIL and P-VMNs at pH 7.4 and pH 6.5 conditions. TRAIL concentration was 100 ng mL^{-1}.

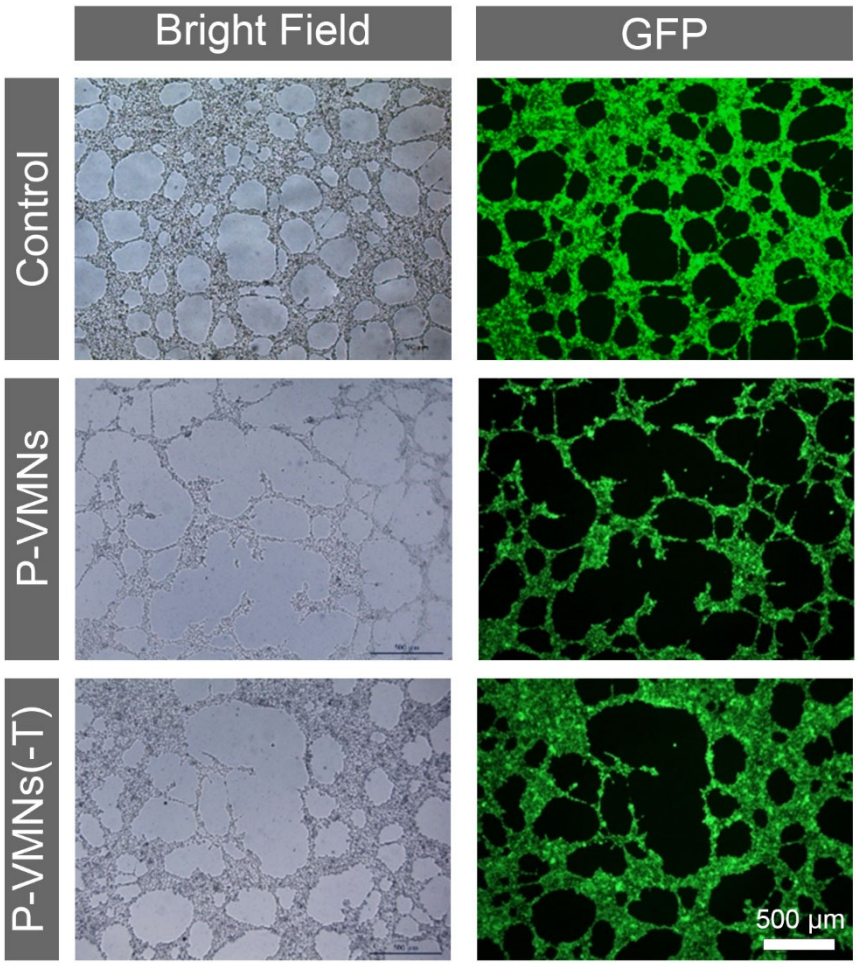


Fig. S18. HUVEC capillary tube formation assay of P-VMNs and P-VMNs(-T).