

Fluorescent Genipin Cross-linked REDV-Conjugated Polymeric Microbubbles for Human Vascular Endothelial Cells (HVECs) Targeting

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Electronic Supplementary Information (ESI)

Materials

Butyl cyanoacrylate (BCA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Triton-100, DMEM and chitosan were purchased from Sigma Aldrich (St. Louis, USA). Acetic acid and ethidium bromide were obtained from Aladdin. REDV peptide was purchased from GL Biochem (Shanghai) Ltd. (Shanghai, China). Dimethyl sulfoxide (DMSO) was purchased from Sigma (St. Louis, MO). pEGFP plasmids (Invitrogen, San Diego, CA) were donated by Dr. Shuai Shi (Wenzhou Medical University Affiliated Eye Hospital). The whole coding sequence of green fluorescent protein (GFP) was inserted into pcDNA3.1 which contains a CMV promoter to construct the plasmid GFP expressing green fluorescent protein. The human endothelial hybridoma cell line EA.hy926 cells and thoracic aorta smooth muscle cells (A7r5) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai). Genipin was purchased from LinChuan Zhixin Biotechnology Co., Ltd. (China).

Methods

Preparation of PBCA Microbubbles (PBCA-MBs)

The synthetic procedure of PBCA-MBs was slightly modified as previously reported.^{1,2} Briefly, Triton-100 (1.5 mL) was added into 150 mL of ultrapure deionized water. The pH of the solution was adjusted to 2.0. BCA monomer (1.5 mL) was added into the solution dropwise when stirring at the speed of 3000 rpm (25 °C). The addition step lasted for around 5 min, and then the reaction was maintained stirring for 1 h by mechanical agitation (10000 rpm) using an Ultra Turrax IKA-25 (IKA Werke, Staufen, Germany). After the emulsification was quenched, centrifugation (3×20 min) at 500 rpm were applied to size-isolate the as-prepared PBCA microbubbles (PBCA-MBs) which were finally collected and dispersed into ultrapure deionized water for storage at 4 °C.

Preparation of Chitosan Surface-coated Microbubbles (PBCA-CS-MBs)

Chitosan was dissolved by 1% acetic acid to prepare a 5 wt% chitosan-acetic acid stock solution. 2 mL of chitosan-acetic acid solution were then added into 30 mL of PBCA-MBs solution. After 2 h of rotational mixing at room temperature, the reaction mixture was centrifuged and washed with ultrapure deionized water for 4 times to collect the PBCA-CS-MBs. The as-prepared microbubbles were then dispersed in deionized water and stored at 4 °C.

Preparation of REDV-conjugated Microbubbles (PBCA-CS-REDV-MBs)

PBCA-CS-REDV-MBs can be synthesized *via* genipin cross-linking and REDV conjugation as reported in the literature earlier.^{3,4} 100 μ L of genipin solution (0.1 mmol/L) and 1 mL of REDV peptide solution (0.01 mmol/L) were added into 5 mL of PBCA-CS-MBs. The mixture reacted for 24 h at 4 °C before transferred into a dialysis bag (molecular weight cut off = 3500 Da). The dialysis bag was laid into a beaker which was filled with ultrapure deionized water, and the excessive peptide and genipin were removed after 48 h at a low stirring speed (500 rpm). The deionized water was renewed every 3 h during the dialysis step. Afterwards, the as-prepared REDV-conjugated microbubbles was centrifuged and collected for characterization and follow-up targeting cell experiments.

Characterization of PBCA-MBs, PBCA-CS-MBs and PBCA-CS-REDV-MBs

The morphology of different as-prepared microbubbles was observed using an inverted fluorescent microscope (Nikon TI-S, Japan). Further detailed morphological characterizations were completed by scanning electron microscope (SEM, Hitachi, Japan) and confocal laser scanning microscope (Ti-E&A1 plus, Nikon, Japan). The excitation and emission spectra were acquired by a spectrofluorometer (FluoroMax-4C-L, Horiba Scientific Instrument Inc., USA). FT-IR spectra were obtained using an FT-IR spectrometer (Bio-Rad FTS-6000, USA). The size and zeta potential were acquired using a Zeta-sizer 3000HS system (Malvern Instrument, Inc., Worcestershire, UK).

Agarose Gel Electrophoresis

Agarose gel electrophoresis was employed as reported previously to assess the DNA condensation capability.⁵ Different microbubbles with varied N/P molar ratios ranging from 0.5 to 20 were prepared. The mixture solution was loaded into the agarose gel (0.8 wt%) containing 0.5 μ g/mL ethidium bromide. Electrophoresis was performed in 1 \times TAE buffer at 100 V for 40 min. UV illuminator was used to indicate the retarded location of the plasmids.

Degradation of Non-targeting (PBCA-MBs) and Targeting (PBCA-CS-REDV-MBs) Microbubbles

To study the degradation behaviour of different as-prepared microbubbles *in vitro*, degradation tests were employed as reported previously.⁶ Microbubbles were placed in closed bottles containing 400 mL of PBS (pH = 7.4). The bottles were incubated in an air-bath shaker at 37 °C for 50 days. The PBS solution was exchanged every 5 days. At different time points, the samples were washed with ultrapure deionized water and dried at room temperature under vacuum until their weight were constantly maintained. After they were weighted, the degradation rates were estimated by the percentage of weight loss. The residual weight (%) was calculated using the following formula: (W_0 : the original weight of microbubble samples; W_t : the sample weight at different time points)

$$\text{Residual weight (\%)} = \frac{W_t}{W_0} \times 100\%$$

Cell Culture

ECs and SMCs were cultured in high glucose DMEM supplemented 10% FBS in 5% CO₂ atmosphere at 37 °C. The non-adherent cells were then discarded, and the adherent cells were cultured to confluence with medium exchanges conducted every 3 days.

In Vitro Cytotoxicity of PBCA-CS-REDV-MBs

In the cytotoxicity study, the cell concentration was constantly maintained as 1×10^6 /mL, and the bubble concentration is 1×10^9 microbubbles/mL. Gradient doses of PBCA-CS-REDV-MBs were incubated with EA.hy926 cells. The cytotoxicity of PBCA-CS-REDV-MBs was evaluated by MTT assay, and the relative cell viability was calculated.⁷ Optical density (OD) was measured by an ELISA reader (Titertek multiscan MC) at the wavelength of 490 nm. The relative cell viability (%) was calculated using the following formula: (OD490': the absorbance value of experimental wells minus zero wells, avg(OD490C'): the average absorbance value of corrected control wells).

$$\text{Relative cell viability} = \frac{OD490'}{\text{avg}(OD490C')} \times 100\%$$

Active Targeting Capability of PBCA-CS-REDV-MBs

ECs and SMCs were added to the culture flask with 5 mL DMEM medium (10% FBS), respectively. Then the cells were mixed gently and co-incubated for 2-3 days until 80-90% confluence. Cells were trypsinized using 0.25% trypsin. After re-suspension, cells were cultured with targeting microbubbles for another 24, 48 and 72 h. Active targeting capability of PBCA-CS-REDV-MBs was observed by an optical microscope at different time points.

Statistical Analysis

All experiments were performed three times. Quantitative data were presented as the Mean \pm SD. Statistical comparisons were analysed using a standard Student's *t*-test. A *p* value of < 0.05 was considered to be statistically significant.

References

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Supporting Figures

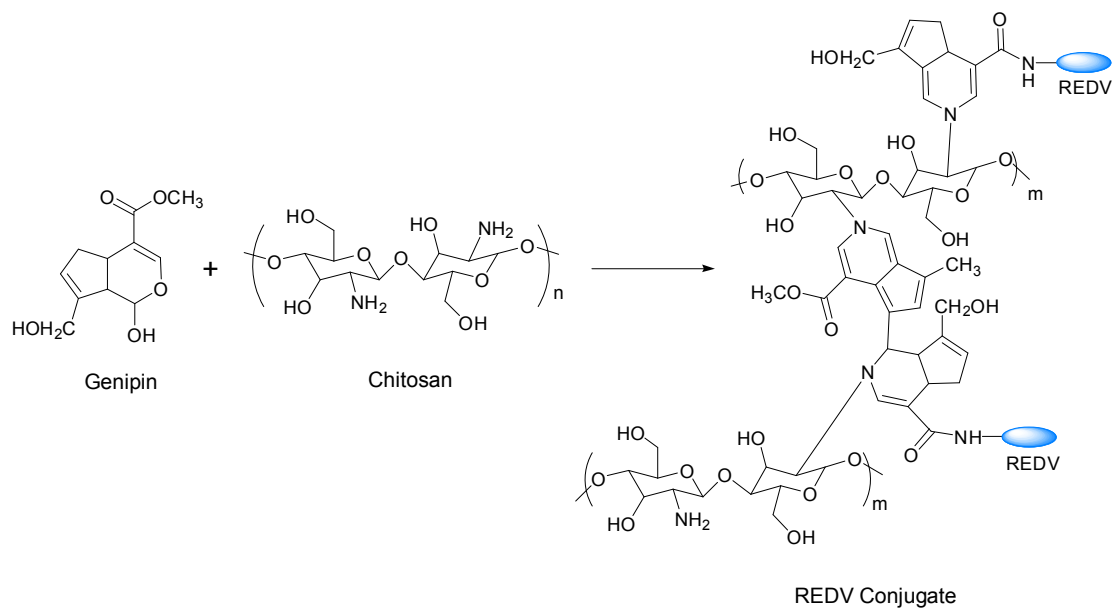


Figure S1. Illustration of chitosan-genipin cross-linking and bio-conjugation of REDV peptides.