Supplementary Material (ESI) for Polymer Chemistry

Supplementary Information

Linear Release Nanoparticle Devices for Advanced Targeted Cancer Therapies with Increased Efficacy

Alice E. van der Ende,[a] Vasanth Sathiakumar,[a] Roberto Diaz,[b]

Dennis E. Hallahan[c] and Eva Harth*[a]

a) Department of Chemistry, Vanderbilt University, 7619 Stevenson Center, Nashville, TN, 37235, USA.

b) Department of Radiation Oncology, Vanderbilt University Medical Center, Nashville, TN, 37232, USA.
Present address: Department of Radiation Oncology, Emory University School of Medicine, 1365 Clifton Road, NE, Suite A1300, Atlanta, GA, 30322, USA.

c) Department of Radiation Oncology, Vanderbilt University Medical Center, Nashville, TN, 37232, USA.
Present address: Department of Radiation Oncology, Mallinckrodt Institute of Radiology; and Siteman Cancer Center, Washington University School of Medicine, St Louis, MO, 63110, USA.

email: eva.harth@vanderbilt.edu

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Materials and Methods

Characterization. ^{1}H NMR spectra were obtained from a Bruker DPX-300 or a Bruker AV-II 600 MHz Fourier Transform Spectrometer, with $CDCl_3$/TMS or DMSO-d_6 as the NMR solvent. Reverse-phase high-performance liquid chromatography (RP-HPLC) was carried out with a Waters HPLC using two Delta-Pak^{TM} PrepLC^{TM} 25 mm Columns (Waters, C18, 300Å, 25 x 100 mm each) with a PrepLC^{TM} 25 mm Radial Compression Module. The products were eluted using a solvent gradient (solvent A = 0.05% trifluoroacetic acid (TFA)/ H_2O; solvent B = 0.05% TFA/ CH_3CN). Accurate molecular mass and purity of the peptides were determined by MALDI-MS, with α-cyano-4-hydroxycinnamic acid as the matrix, on a Perspective Biosystems Voyager-DE STR (Framingham, MA) equipped with delayed extraction technology operating in reflector mode. For dynamic light scattering (DLS), a Malvern Nano ZS system by Malvern Instruments (Malvern Zetasizer Nanoseries, Malvern, UK) was employed at a fixed angle of 90º at 25 ºC, taking the average of three measurements. The particles were diluted with toluene to a concentration, which gave the desired number of counts in order to obtain a good signal-to-noise ratio. Static light scattering was also performed on the Malvern Nano ZS to obtain the absolute weight average molecular weights of the nanoparticles. Different sample concentrations (0.5-1.2 mg/mL) were prepared by dilution of a high concentration stock solution in toluene (3 mg/mL). Data collection and calculations were managed using the Molecular Weight function in the DTS software for the Nano ZS system, which compiles the static intensity measurements, generates a standard Debye plot, and then calculates the weight average molecular weight. Samples for transmission electron microscopy (TEM) imaging were prepared by dispersing 0.5 mg nanoparticles in 1 mL Lonza cell culture water. The samples were sonicated for 5 min and were stained with 6 drops of 3% phosphotungstic acid. The carbon grids were prepared by slowly dipping an Ultrathin Carbon Type-A 400 Mesh Copper Grid (Ted Pella, Inc., Redding, CA)

into the particle solutions three times and drying the grid at ambient temperature. A Philips CM20 transmission electron microscope operating at 200 kV in bright-field mode was used to obtain TEM micrographs of the polymeric nanoparticles. UV-Vis measurements were made via a Thermo Scientific NanoDrop^{TM} 1000 spectrophotometer (Wilmington, DE).

Materials. Reagent chemicals were purchased from Aldrich (St. Louis, MO) and Acros (Morris Plains, NJ), and used as received, unless otherwise stated. Spectra/Por® Dialysis membrane and SnakeSkin® Pleated Dialysis Tubing, regenerated cellulose, were obtained from Spectrum Laboratories Inc. (Rancho Dominguez, CA) and Pierce Biotechnology (Thermo Fisher Scientific, Rockford, IL), respectively. Fmoc protected amino acids were obtained from Advanced ChemTech (Louisville, KY). Analytical TLC was performed on commercial Merk plates coated with silica gel GF254 (0.24 mm thick). Silica gel for column chromatography was Merck Kieselgel 60 (230-400 mesh, ASTM). All monomers and polymers were synthesized according to the literature.[8, 9]

General procedure for the formation of nanoparticles from poly(vl-evl). In a 100 mL three-necked round bottom flask equipped with stir bar, condenser and septa, 2,2'-(ethylenedioxy)bisethylamine (34.1 μL, 2.32 x10^{-4} mol), 28.7 mL CH_2Cl_2 and a solution of poly(vl-evl) (0.14 g, M_w= 3400 Da, PDI = 1.16) in 0.19 mL CH_2Cl_2 were added. The mixture was heated at 44 ºC for 12 h. Residual diamine was removed by dialyzing with SnakeSkin® Pleated Dialysis Tubing (MWCO = 10,000) against dichloromethane. DLS: D_H = 272.3 ± 23.3 nm. ^{1}H NMR (300 MHz, $CDCl_3$/TMS) δ: The significant change is the disappearance of the epoxide protons at 2.94, 2.75 and 2.47 ppm and the appearance of signals at 3.64 and 2.97 ppm corresponding to the protons neighboring the secondary amine of the PEG linker after cross-linking. All other aspects of the spectrum are similar to that of poly(vl-evl), as referenced in the literature.[8]

General procedure for *in vitro* nanoparticle degradation studies. Poly(vl-evl) nanoparticles (10 mg) were suspended in 2 mL of Dulbecco's Phosphate Buffered Saline (pH 7.2) in 2 dram vials equipped with stir bars. The vials were sealed to avoid evaporation and the samples were maintained at 37 °C under continuous stirring. At 48 h intervals, samples were removed and dichloromethane was added (3 x 4 mL) to extract remaining nanoparticles and degradation products. The extraction solutions were concentrated via rotary evaporator and dried *in vacuo*. The degradation of the nanoparticles was monitored by the change in molecular weight, as determined by static light scattering, with incubation time.

General procedure for nanoparticle formation from poly(vl-evl-avl-opd). To a solution of 2,2'-(ethylenedioxy)diethylamine (23.4 μL, 0.16 mmol) in CH_2Cl_2 (98.7 mL), a solution of poly(vl-evl-avl-opd) (0.1840 g, M_w= 3440 Da) in CH_2Cl_2 (0.64 mL) was added. The mixture was heated at 44 °C for 12 h. Residual diamine was removed by dialyzing with SnakeSkin® Pleated Dialysis Tubing (MWCO = 10,000) against CH_2Cl_2. Yield: 0.15 g (94%). DLS: D_H = 52.9 ± 3.3 nm. SLS: M_w = 147,000 Da. ^{1}H NMR (300 MHz, $CDCl_3$/TMS) δ: The significant change is the disappearance of the epoxide protons at 2.94, 2.75 and 2.47 ppm and the appearance of signals at 3.54 and 2.97 ppm corresponding to the protons neighboring the secondary amine of the PEG linker after cross-linking. All other aspects of the spectrum are similar to that of poly(vl-evl-avl-opd), as referenced in the literature.[9]

Formulation of poly(vl-evl-avl-opd) nanoparticles with TPGS-vitamin E. To a 150 mL beaker containing $_D$-α-tocopheryl polyethylene glycol 1000 succinate (vitamin E TPGS) (0.28 g) dissolved in Lonza cell culture water (55 mL), nanoparticles (0.0977g) dissolved in dimethyl sulfoxide (DMSO) (0.50 mL) were added slowly with vigorous stirring. The solution was split equally

into two 50 mL centrifuge tubes. The nanoparticles were rinsed by applying three cycles of centrifugation (8000 rpm for 30 min) and reconstituted with cell culture water. The nanoparticle suspension was then lyophilized.

***In vitro* poly(vl-evl-avl-opd) nanoparticle degradation studies.** TPGS formulated poly(vl-evl-avl-vl) nanoparticles (10 mg) were suspended in 2 mL of Dulbecco's Phosphate Buffered Saline (pH 7.4) in 2 dram vials equipped with stir bars. The vials were sealed to avoid evaporation and the samples were maintained at 37 ºC under continuous stirring. At 48 h intervals, samples were removed and dichloromethane was added (3 x 4 mL) to extract remaining nanoparticles and degradation products. The extraction solutions were concentrated via rotary evaporator and dried *in vacuo*. The degradation of the nanoparticles was monitored by the change in molecular weight, as determined by static light scattering, with incubation time.

***In vitro* cytotoxicity of formulated poly(vl-evl-avl-opd) nanoparticles (MTT assay).** The cytotoxicity of TPGS formulated nanoparticles was evaluated using an MTT assay. HeLa cells were cultured in Eagle's Minimum Essential Medium supplemented with 10% heat inactivated fetal bovine serum, L-glutamine, penicillin streptomycin sulfate antibiotic-antimycotic mixture and gentamicin. Cells were maintained at 37 ºC with 5% CO_2 in a 95% humidity incubator. The cells were seeded in a 96-well plate in 100 µL media per well at a density of 10,000 cells/well and incubated for 24 h. The media was then replaced with 100 µL of phenol red free medium-containing nanoparticles at different concentrations in triplicate and incubated for 24 h. After incubation, the nanoparticle containing media were removed, the cells were rinsed three times with DPBS, to avoid interference in the assays, and 100 µL of fresh phenol red free media was added, followed by 10 µL MTT solution (5 mg/mL). The cells were incubated for 4 h, after which time the medium was carefully removed. To the resulting

purple crystals, 100 μL DMSO was added to lyse the cells and was incubated for 10 min at 37ºC. The MTT absorbance was measured at 540 nm using a Synergy HT Multi-mode microplate reader (Bio Tek Instruments, Winooski, VT). Optical densities measured for wells containing cells that received no nanoparticle were considered to represent 100% viability. Results are expressed as the mean±S.D. of viable cells.

In vitro **release of paclitaxel from poly(vl-evl-avl-opd) nanoparticles.** To a 150 mL beaker containing $_{D}$-α-tocopherol polyethylene glycol 1000 succinate (0.34 g) dissolved in Lonza cell culture water (68 mL), poly(vl-evl-avl-opd) nanoparticles (56.5 mg) and paclitaxel (8.5 mg) dissolved in dimethyl sulfoxide (0.50 mL) were added slowly with vigorous stirring. The solution was split equally into two 50 mL centrifuge tubes. The paclitaxel loaded nanoparticles were purified by applying three cycles of centrifugation (8000 rpm for 30 min) and reconstituted with cell culture water. The nanoparticle suspension was then lyophilized. The concentration of encapsulated paclitaxel was determined by NanoDrop^{TM} UV-Vis at a wavelength of 254 nm. Paclitaxel standards (0.398-2.39 mg/mL) were measured by UV-Vis and a calibration curve was rendered. With the calibration curve, the concentration of encapsulated paclitaxel was determined by the absorbency of the paclitaxel in the nanoparticle at 254 nm and the loading ratio was found to be 11.3%. The release of paclitaxel from the nanoparticles was measured in PBS (pH 7.4) at 37 °C. The paclitaxel-loaded nanoparticles (20 mg) were suspended in PBS (20 mL). At particular time intervals, the nanoparticle dispersion was centrifuged, the supernatant was removed and the released paclitaxel was extracted from the supernatant with CH_2Cl_2. The concentration of released paclitaxel was determined by NanoDrop^{TM} UV-Vis at a wavelength of 254 nm as mentioned above.

General procedures for the synthesis of HVGGSSV peptide. The peptide was synthesized by solid-phase peptide synthesis using standard Fmoc chemistry on a Model 90 Peptide Synthesizer (Advanced ChemTech).

General procedure: Attachment of N-Fmoc amino acids to resin. After swelling with dichloromethane (20 mL) for 20 min, H-Val-2-Cl-Trt resin (0.20 g, 1.03 mmol/g, 0.21 mmol surface amino acids) was treated with a solution of Fmoc-protected amino acids (4 equiv, 0.9 mmol) in dimethylformamide (DMF) (6 mL). The amino acids were attached to the resin using double coupling with a solution (9 mL) consisting of N-hydroxybenzotriazole monohydrate (HOBt) (0.9 mmol, 137.8 mg) *o*-(benzotriazole-N,N,N',N'-tetramethyluronium hexafluorophosphate (0.9 mmol, 0.34 g), N,N'-diisopropylethylamine (DIPEA) (1.8 mmol, 0.31 mL) in 9 mL DMF. The reaction mixture was shaken for 60 min and washed with DMF (4 x 10 mL), methanol (4 x 10 mL) and DMF (4 x 10 mL). A 20% (v/v) piperidine in DMF solution was used to deprotect the Fmoc groups. The amino acids were attached to the resin in the following sequence: Ser, Ser, Gly, Gly, Val, His, Asn, Gly, Gly, Gly, Cys, and Gly.

General procedure: Cleavage from resin. The resin was treated with Reagent R, a solution of TFA, thioanisole, anisole, and ethanedithiol (90:5:3:2, 6 mL), for 4 h. After removal of the resin by filtration, the filtrate was concentrated to precipitate the peptide with cold diethyl ether. Crude peptides were purified by RP-HPLC and lyophilized. Peptide identity was confirmed by MALDI-MS (*m/z*: 1086.45).

Attachment of HVGGSSV peptide to nanoparticles. To a solution of nanoparticles (105.6 mg, 0.78 μmol) in DMSO (1 mL), HVGGSSV peptide (56 mg, 53.6 μmol) in DMSO (2 mL) was added. The reaction mixture was heated for 72 h at 34 °C. Residual peptide was removed by dialyzing with SnakeSkin® Pleated Dialysis Tubing (MWCO = 10,000) against 50/50 THF/CH_3CN. Yield: 77

mg. DLS: D_H = 55.3 ± 3.6 nm; original particle D_H = 52.9 ± 3.3 nm. SLS: M_w = 185,000 Da; original particle M_w = 147,000 Da. ^{1}H NMR (600 MHz, DMSO-d_6) δ: The significant change is the reduction of the allyl protons at 5.69 and 5.00 ppm and the appearance of signals at 0.80, 1.39, 1.65, 2.74, 3.07, 3.75, 4.40 and 7.11-8.32 ppm due to the peptide. All other aspects of the spectrum are similar to that of the poly(vl-evl-avl-opd) nanoparticles, as referenced in the literature.[9]

Encapsulation of paclitaxel in HVGGSSV conjugated poly(vl-evl-avl-opd) nanoparticles. To a 150 mL beaker containing $_D$-α-tocopherol polyethylene glycol 1000 succinate (0.30 g) dissolved in Lonza cell culture water (60 mL), HVGGSSV-nanoparticles (0.0681 g) and paclitaxel (10.2 mg) dissolved in dimethyl sulfoxide (0.50 mL) were added slowly with vigorous stirring. The solution was split into two 50 mL centrifuge tubes. The paclitaxel loaded nanoparticles were purified by applying two cycles of centrifugation (8000 rpm for 30 min) and reconstituted with cell culture water. The nanoparticle suspension was then lyophilized. The loading ratio of paclitaxel for the encapsulation was determined by NanoDrop™ UV-Vis at 254 nm as mentioned above and was found to be 11%.

Figure 4a. Drug Release in comparison to other polyester based nanoparticle systems.