Shell-crosslinked knedel-like nanoparticles for delivery of cisplatin: Effect of crosslinking

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Experimental Section

Materials. Cisplatin was purchased from Strem Chemicals, Inc. (Newburyport, MA). All other chemicals were purchased from Aldrich Chemical Co. (St. Louis, MO) and used without further purification unless otherwise noted. Slide-A-Lyzer dialysis cassettes (10 kDa molecular weight cut-off, MWCO) were purchased from Pierce Biotech. (Rockford, IL). The Spectra/Por Dialysis Membranes (MWCO 6-8 kDa) were purchased from Spectrum Laboratories, Inc (Rancho Dominguez, CA). Nanopure water (18 MΩ·cm) was acquired by means of a Milli-Q water filtration system, Millipore Corp. (Bedford, MA).

Instrumentation. ^{1}H NMR and ^{13}C NMR spectra were recorded on Inova 300 or Mercury 300 spectrometer interfaced to a UNIX computer using VnmrJ software. Chemical shifts were referenced to the solvent residual signals.

FTIR spectra were recorded on an IR Prestige 21 system using a diamond ATR lens (Shimadzu Corp., Japan) and analyzed using IRsolution v. 1.40 software.

Gel permeation chromatography (GPC) was performed on a Waters Chromatography, Inc., 1515 isocratic HPLC pump equipped with an inline degasser, a model 2414 differential refractometer (Waters, Inc.), and four PLgel polystyrene-*co*-divinylbenzene gel columns (Polymer Laboratories, Inc.) connected in series: 5 μm Guard (50×7.5 mm), 5 μm Mixed C (300×7.5 mm), 5 μm 10^4 (300×7.5 mm), and 5 μm 500 Å (300×7.5 mm). The instrument was operated at 40 °C with tetrahydrofuran (THF) as eluent (flow rate set to 1.0 mL/min). Polymer solutions were prepared at a known concentration (*ca.* 3 mg/mL) and an injection volume of 200 μL was used. Data collection and analysis was performed with the Breeze (version 3.30, Waters, Inc.) software. The system was calibrated with polystyrene standards (Polymer Laboratories,

Amherst, MA).

Dynamic light scattering (DLS) measurements were conducted using Delsa Nano C (Beckman Coulter, Inc., Fullerton, CA) equipped with a laser diode operating at 658 nm. Size measurements were made in nanopure water (n = 1.3329, η = 0.890 cP at 25 ± 1 °C). Scattered light was detected at 165° angle and analyzed using a log correlator over 70 accumulations for a 3.0 mL sample in a glass sizing cell (4.0 mL capacity). The photomultiplier aperture and the attenuator were automatically adjusted to obtain a photon counting rate of ca. 10 kcps. Calculation of the particle size distribution and distribution averages was performed using CONTIN particle size distribution analysis routines. The peak averages of histograms from number distributions out of 70 accumulations were reported as the average diameters of the particles.

Glass transition temperatures (T_g) were measured by differential scanning calorimetry on a Mettler-Toledo DSC822® (Mettler-Toledo, Inc., Columbus, OH), with a heating rate of 10 °C/min. Measurements were analyzed using Mettler-Toledo Star^e v. 7.01 software. The T_g was taken as the midpoint of the inflection tangent, upon the third heating scan. Thermogravimetric analysis was performed under N_2 atmosphere using a Mettler-Toledo model TGA/SDTA851^e, with a heating rate of 10 °C /min. Measurements were analyzed by using Mettler-Toledo Stare v. 7.01 software.

Transmission electron microscopy (TEM) images were collected on a JEOL 1200EX operating at 100 kV and micrographs were recorded at calibrated magnifications using a SIA-15C CCD camera. A FEI Tecnai G2 F20 ST operated at 200 kV was also adopted to obtain high resolution TEM (HR-TEM) image, as well as the high-angle annular dark field imaging in the scanning TEM mode (HAADF-STEM). The samples as aqueous solutions (4 μL) were

deposited onto carbon-coated copper grids, which were pre-treated by a glow discharge on a PELCO easiGlow™ to increase the surface hydrophilicity. Excess sample was wicked off using filter paper and the grids were allowed to dry in air overnight. For samples without platinum loading, a drop of 1% phosphotungstic acid (PTA) stain was then added, and allowed to stand for 30 seconds before excess stain was wicked away.

The concentration of platinum was measured by inductively coupled plasma-mass spectrometry (ICP-MS) on Perkin Elmer DRCII ICP-MS. The element/mass detected was ^{195}Pt and the internal standard used was ^{193}Hf.

Preparation of poly(*tert*-butyl acrylate), P*t*BA$_{90}$.

A flame-dried 100-mL Schlenk flask equipped with a magnetic stir bar was charged with S-dodecyl-S′-(α,α′-dimethyl-α″-acetic acid) trithiocarbonate (DDMAT, 0.547 g, 1 *eq*.), *tert*-butyl acrylate (*t*-BA, 57.7 g, 300 *eq*.), azobisisobutyronitrile (AIBN, 12.3 mg, 0.05 *eq*.), and 2-butanone (27 mL). The flask was sealed with a rubber septum and allowed to stir for 10 min at room temperature to ensure homogeneous mixing. The reaction mixture was degassed by several freeze-pump-thaw cycles (N>3), after which the flask was allowed to return to room temperature and was allowed to stir for an additional 10 min. The flask was then immersed into a pre-heated oil bath at 52 °C to start the polymerization. The polymerization was monitored by analyzing aliquots collected at pre-determined times by ^{1}H-NMR spectroscopy. As the expected monomer conversion was reached, after *ca*. 3 h depending on the desired block chain lengths, the polymerization was quenched by quick immersion of the reaction flask into liquid N_2 and opening to air. THF (20 mL) was added to the reaction flask and the polymer was purified by precipitation into 2 L of a methanol/ice (1:1) mixture four times. The precipitants were collected

and dried under vacuum overnight to afford the final product as a yellow powder (13.6 g, 31.3% conversion, 73% yield). GPC: M_n = 12400 Da, PDI = 1.11. IR: 3026–2885, 1720, 1450, 1365, 1250, 1142, 841, 749 cm^{-1}. ^{1}H NMR ($CDCl_3$, 300 MHz): δ 0.86 (t, *J* = 6 Hz, *C**H**$_3$*CH_2–), 1.13–1.90 (br, –CH*C**H**$_2$*– of the polymer backbone, alkyl chain of initiator, and HOOCC(C***H***$_3$)$_2$–), 1.28–1.58 (br, C***H***$_3$C), 2.12–2.38 (br, –C***H***CH_2– of the polymer backbone), 3.31 (t, *J* = 2 Hz, –SCSC***H***$_2$–), 4.62–4.72 (br, –CH_2C***H***S) ppm. ^{13}C NMR ($CDCl_3$, 75 MHz): δ 28.2, 34.0–38.5, 41.1–42.9, 80.1–81.0, 173.6–174.6 ppm. DSC: T_g = 47 °C. TGA in N_2: 200–260 °C, 47% mass loss; 260–480 °C, 41% mass loss, 12 % mass remaining above 480 °C.

Preparation of poly(*tert*-butyl acrylate)-*b*-polystyrene, P*t*BA$_{90}$-*b*-PS$_{120}$.

To a flame-dried 50 mL Schlenk flask equipped with a magnetic stir bar, P*t*BA_{90} (2.48 g, 1 *eq*.), styrene (16.6 g, 798 *eq*. based on desired polymer chains), AIBN (1.6 mg, 0.05 *eq*.) and 1,4-dioxane (4.2 mL) were added. The flask was sealed with a rubber septum and allowed to stir for 10 min. The reaction mixture was then degassed by several freeze-pump-thaw cycles (N>3). After allowing the flask to return to room temperature, it was allowed to stir in a pre-heated oil bath at 60 °C to start the polymerization. The polymerization was monitored by analysis of aliquots taken at various times by ^{1}H-NMR spectroscopy. The polymerization was quenched at *ca*. 50 h (depending on desired block chain lengths) by immersing the flask in liquid nitrogen and opening to air. The product was purified by precipitation into methanol/water (5:1) mixture three times and dried under vacuum overnight to yield a pale yellow powdery product (4.85 g, 16.7% conversion, 92% yield). GPC: M_n = 25300 Da, PDI = 1.13. IR: 3109–2792, 1728, 1489, 1450, 1365, 1250, 1149, 848, 756 cm^{-1}. ^{1}H NMR (CD_2Cl_2, 300 MHz): δ 0.89 (t, *J* = 6 Hz, *C**H**$_3$*CH_2–), 1.15–2.13 (br, –CH*C**H**$_2$*– of the polymer backbone, alkyl chain of initiator, and

HOOCC(C***H***$_3$)$_2$-), 1.12–1.64 (br, C***H***$_3$C), 2.12–2.36 (br, C_5H_5C***H***CH_2– of the polymer backbone), 3.20–3.32 (br, -SCSC***H***$_2$–), 6.34–7.56 (br, Ar–H) ppm. ^{13}C NMR (CD_2Cl_2, 75 MHz): δ 28.4, 36.1–38.2, 40.6–43.0, 80.9, 125.8–129.4, 145.2-147.0, 174.4–175.2 ppm. DSC: $(T_g)_1$ = 48 °C, $(T_g)_2$ = 102 °C. TGA in N_2: 190–270 °C, 16% mass loss; 270–470 °C, 54% mass loss, 30 % mass remaining above 470 °C.

Preparation of poly(acrylic acid)-*b*-polystyrene, PAA_{90}-*b*-PS_{120}.

A flame-dried 100 mL round bottom flask equipped with a magnetic stir bar was charged with P*t*BA_{90}-*b*-PS_{120} (1.00 g) and dichloromethane (4 mL). Trifluoroacetic acid (TFA, 47 mL, 162 *eq*.) was added to the stirring solution and the reaction was allowed to stir 24 h at room temperature, after which the solvent was removed under vacuum. The crude product was resuspended in 20 mL of THF and transferred to a pre-soaked dialysis tubing (MWCO *ca*. 6–8 kDa), and dialyzed against nanopure water for 3 days. The solution was then lyophilized to yield a yellowish solid (0.791 g, 95% yield). IR: 3600–2400, 1708, 1492, 1451, 1238, 1177, 1067, 1029, 754, 696 cm^{-1}. ^{1}H NMR (DMSO-d_6, 300 MHz): δ 1.02-1.08 (m, *C*H$_3$CH$_2$–), 1.00–2.02 (br, –CH*C*H$_2$– of the polymer backbone, alkyl chain of initiator, and HOOCC(C***H***$_3$)$_2$-), 2.09–2.39 (br, C_5H_5C***H***CH_2– of the polymer backbone), 2.71–2.73 (br, –SCSC***H***$_2$–), 6.29–7.39 (br, Ar–H) ppm. ^{13}C NMR (DMSO-d_6, 75 MHz): δ 34.2–36.8, 40.1–41.6, 124.7–128.6, 144.8-145.8, 175.5-176.0 ppm. DSC: $(T_g)_1$ = 103 °C, $(T_g)_2$ = 145 °C. TGA in N_2: 190–290 °C, 5% mass loss; 290–490 °C, 69% mass loss 26 % mass remaining above 490 °C.

Preparation of micelles and shell crosslinked knedel-like (SCK) nanoparticles.

Micelles: PAA_{90}-*b*-PS_{120} (*ca*. 25 mg) was dissolved in DMF (25 mL) in a 100 mL round bottom flask and allowed to stir for 30 min at room temperature. To this solution, an equal volume of nanopure water was added dropwise *via* a syringe pump over a period of 2 h. The reaction mixture was allowed to stir overnight at room temperature and dialyzed against nanopure water for 2 days in a presoaked dialysis tubing (MWCO *ca.* 6–8 kDa) to afford a micelle solution with a final polymer concentration of *ca.* 0.22 mg/mL.

SCKs: To the micelle solution of PAA-*b*-PS was added a solution of 2,2′-(ethylenedioxy)diethylamine (EDDA) in nanopure water (*ca.* 2 mg/mL, 0.05 or 0.15 *eq*., with respect to acid residues, nominal 10% or 30% crosslinking, respectively) dropwise, and the solution was allowed to stir for 2 h. To this solution, 1-[3´-(dimethylamino)propyl]-3-ethyl-carbodiimide methiodide (EDCI) in nanopure water (*ca.* 1 mg/mL, 0.15 or 0.30 *eq*., with respect to acid residues) was added dropwise over 20 min and the resulting mixture was allowed to stir overnight before dialysis against nanopure water for 2 days in presoaked dialysis tubing (MWCO *ca*. 6–8 kDa) to afford SCK solutions with a final polymer concentration of *ca*. 0.22 mg/mL.

Loading cisplatin into micelles and SCK nanoparticles.

In a typical experiment, to a vial containing a magnetic stir bar and micelle or SCK solution (10 mL, polymer PAA_{90}-*b*-PS_{120} concentration 0.22 mg/mL, acid residues had been neutralized by addition of 0.1 M $NaOH_{(aq)}$), a solution of cisplatin (1.0 mg/mL in water, 0.5 molar ratio with respect to the PAA carboxylate residues) was added dropwise. The solution was stirred for three days and then dialyzed against nanopure water at room temperature to remove free cisplatin. Dynamic light scattering (DLS), transmission electron microscopy and inductively coupled

plasma mass spectrometry (ICP-MS) were used to measure the particle dimensions and platinum concentrations, respectively (see manuscript Figures 1 and 2, and Table 1).

Release studies of Cisplatin-loaded micelles and SCK nanoparticles.

In a typical procedure, a solution of CDDP-loaded micelles or SCKs (0.9 mL) was transferred into a presoaked dialysis cassette (Slide-A-Lyzer, 10 kDa MWCO, Pierce Biotechnology, Rockford IL). The cassette was allowed to stir in a beaker containing PBS (180 mL, 10 mM phosphate, 137 mM NaCl, pH 7.4 at 37 ^{o}C), and samples (2 mL) were collected from the dialysate at pre-determined times and analyzed by ICP-MS, with fresh PBS (2 mL) added back into the beaker.

***In vitro* cytotoxicity assays.**

OVCAR-3 ($5x10^3$ cells/well) or RAW 264.7 ($2x10^4$ cells/well) cells were plated in a 96-well plate in RPMI media and Dulbecco's Modified Eagle Medium (DMEM) (20% and 10% fetal bovine serum, for the OVCAR-3 and RAW 264.7, respectively, and 1% penicillin/streptomycin). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h to adhere. Then, the medium was replaced with a fresh media 1 h prior to the addition of the various formulations at concentrations that ranged from $7x10^{-4}$ to 150 μM of cisplatin. For each well, 20 μL of the formulations was added to 100 μL of the medium. The cells were incubated with the formulations for 72 h and washed once with PBS and 100 μL of the complete media was added to the cells. MTS combined reagent (20 μL) was added to each well (Cell Titer 96® Aqueous Non-Radioactive Cell Proliferation Assay, Promega Co., Madison, WI). The cells were incubated with the reagent for 2 h at 37 °C in a humidified atmosphere containing 5% CO_2

protected from light. Absorbance was measured at 490 nm using SpectraMax M5 (Molecular Devices Co., Sunnyvale, CA). The cell viability was calculated based on the relative absorbance to the control untreated cells. The IC_{50} values were calculated using GraphPad Prism four-parameter fit, considering the 0% and 100% viabilities are for media control (no cells) and cells with no treatment, respectively.

Multiplex assay

RAW 264.7 ($2x10^4$ cells/well) cells were plated in a 96-well plate in DMEM, as described in the previous section. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h to adhere. Then, the medium was replaced with a fresh media 1 h prior to the treatment with medium (control), micelles and SCKs of varying degrees of crosslinking (10 and 30%) at concentrations of 32 μg/mL. The supernatants were then collected and centrifuged for 10 min at 13,000 rpm. Serial dilutions of standards of cytokines were also prepared in the same diluent utilized for the samples (*i.e.* cell-culture medium). Control, standards and nanoparticle-treated samples (50 μL) were incubated with antibody-conjugated magnetic beads for 30 min in the dark. After washing, the detection antibody was added to the wells and incubated in the dark for 30 min under continuous shaking (300 rpm). After washing, streptavidin-phycoerythrin was added to every well and incubated while protected from light for 10 min under the same shaking conditions. Finally, after several washings and re-suspension in the assay buffer and shaking, the expression of the mouse cytokines, interleukin (IL)-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (P40), IL-12 (P70), IL-13, IL-17, Eotaxin, granulocyte-colony-stimulating factor (G-CSF), granulocyte macrophage-colony-stimulating factor (GM-CSF), interferon-γ (IFN-γ), keratinocyte-derived chemokine (KC), monocyte chemotactic protein (MCP)-1, macrophage

inflammatory protein (MIP)-1α, MIP-1β, regulated upon activation normal T-cell expressed and presumably secreted (RANTES) and tumor necrosis factor-α (TNF-α) was measured immediately using Bio-plex 200 system with HTF and Pro II Wash station and the data were analyzed using the Bio-plex Data Pro software (Bio-Rad Laboratories, Inc., Hercules, CA).

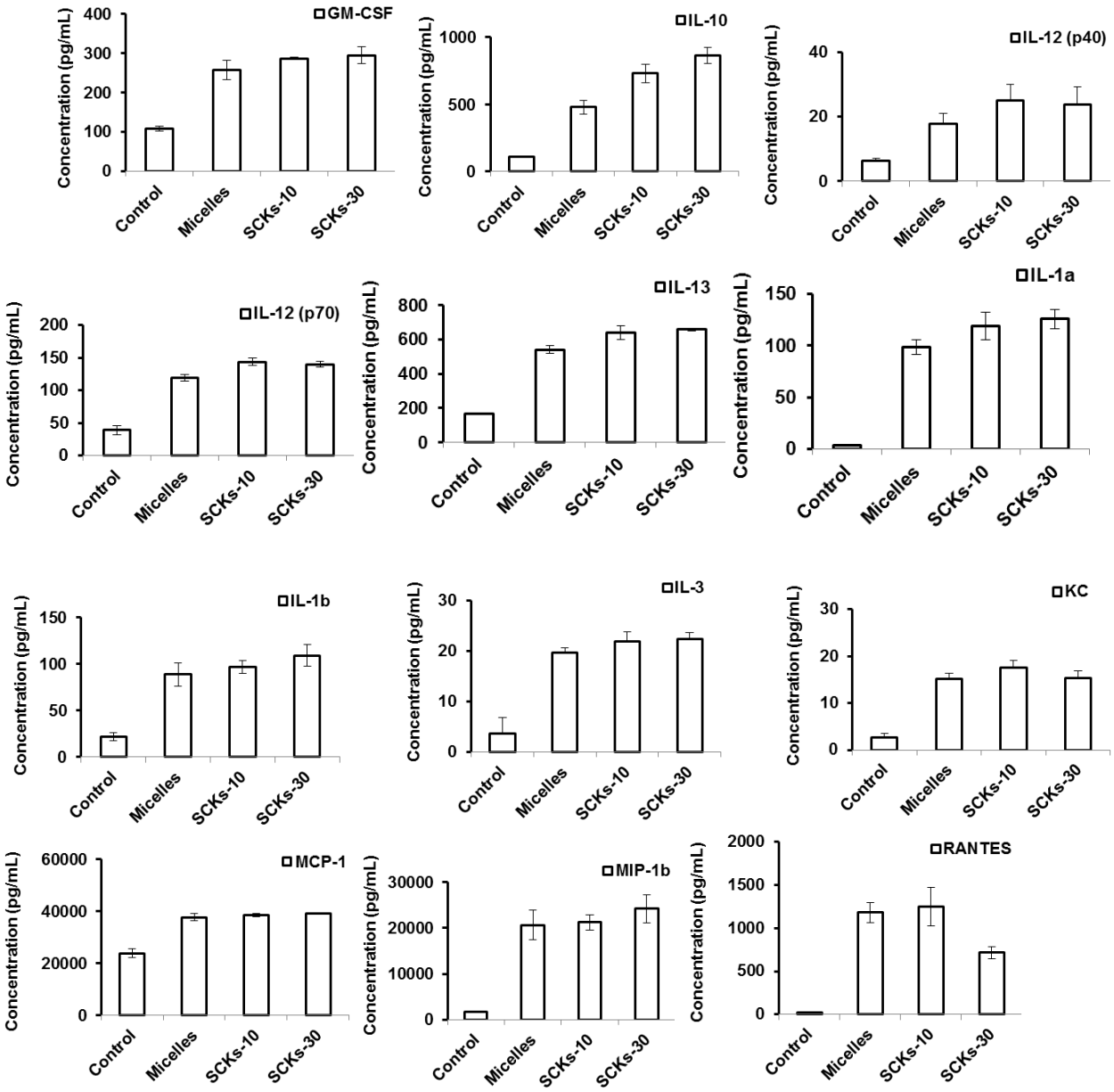

Figure S1. The expression of the mouse cytokines, GM-CSF, IL-3, IL-10, IL-12 (P40), IL-12 (P70), IL-13, IL-1α, IL-1β, KC, MCP-1, MIP-1β and RANTES following the treatment of RAW 264.7 cells with media (control), micelles and SCKs of varying degrees of crosslinking (10 and 30%) at 32 μg/mL.