Supporting Information

Inhibitory Effects of Multivalent Polypeptides on the Proliferation and Metastasis of Breast Cancer Cells

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Materials

Boc-Tyr(tBu)-OH, MeO-Ser(tBu)-H•HCl, MeO-Leu-H•HCl, Boc-Lys(Boc)-OH, MeO-Lys-H•2HCl, 1-ethyl-3-(3-dimethylaminopr-opyl) carbodiimide hydrochloride (EDC•HCl), benzotriazole-1-yloxytrripyrrolidino phosphonium hexafluorophosp-hate (PyBOP) and 1-hyd-roxybenzotriazole hydrate (HOBt) were purchased from GL Biochem LLC (Shanghai, China). AstaTech Pharmaceuticals (Chengdu, China) provided diisopropylethylamine (DIPEA) and trifluoroacetic acid (TFA). N, N-dimethylformamide (DMF, chromatographically pure) was acquired from Kemiou Chemical Reagent Company (Tianjin, China). Fluorescein isothiocyanate (FITC) were acquired from Sigma-Aldrich (United States of America). Taxol was purchased from the Taiji Group (Chongqing, China).

Apoptosis Detection Kits (Annexin FITC/PI) and Hoechst 33342 were purchased from Dojindo Laboratories (Japan). MTT reagent (3-(4, 5-dimethyl-2-thiazol)-2, 5-diphenyl-2H-tetrazolium bromide) was purchased from Solarbio (Beijing, China). Phosphate buffered saline (PBS), Fluorescein diacetate (FDA), and propodium iodide (PI) were acquired from Sigma-Aldrich (USA). Crystal violet and Solarbio (Beijing, China) provided gelatin. Matrigel was purchased from Corning (BD Biocoat, USA). Alexa Fluor™ 488 phalloidin was acquired from ThermoFisher Scientific. Cell cycle kits (RNase A/PI) were purchased from KeyGEN BioTECH (Jiangsu China).

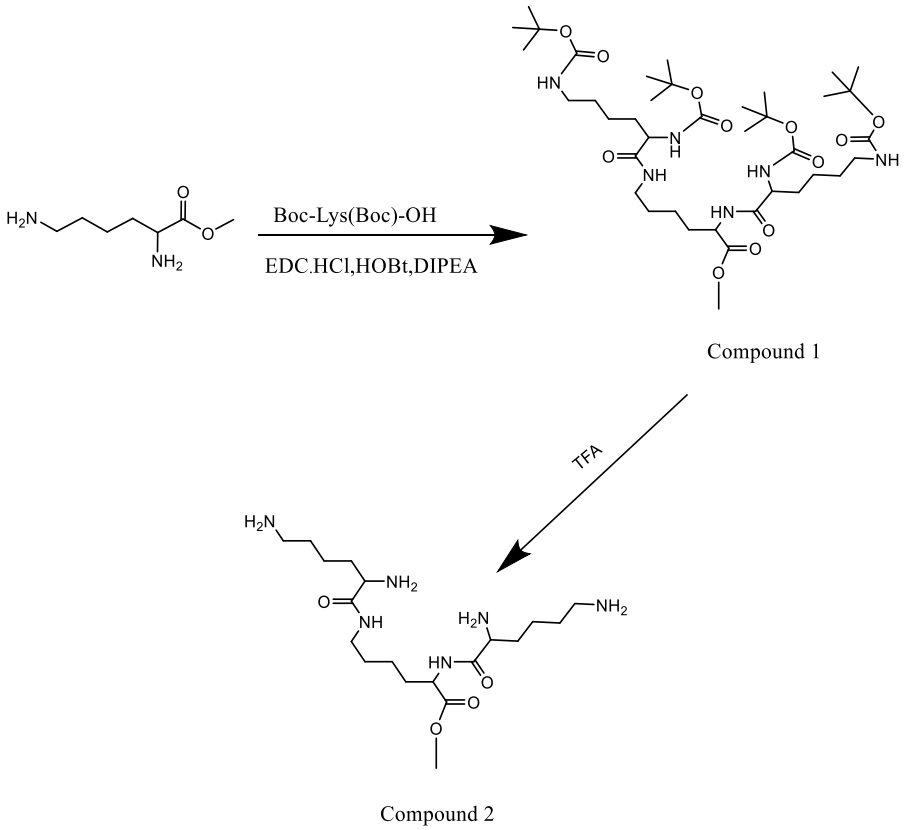
Mouse breast cancer cells (4T1) and mouse fibroblast cells (L929) were purchased from the Chinese Academy of Science Cell Bank for Type Culture Collection (Shanghai, China). RPMI-1640, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin and streptomycin were obtained from Gibco (USA). Cells were cultured in RPMI-1640 containing 10% FBS, 1% penicillin and 1% streptomycin at 37 °C and 5 % CO_2.

Experimental Details

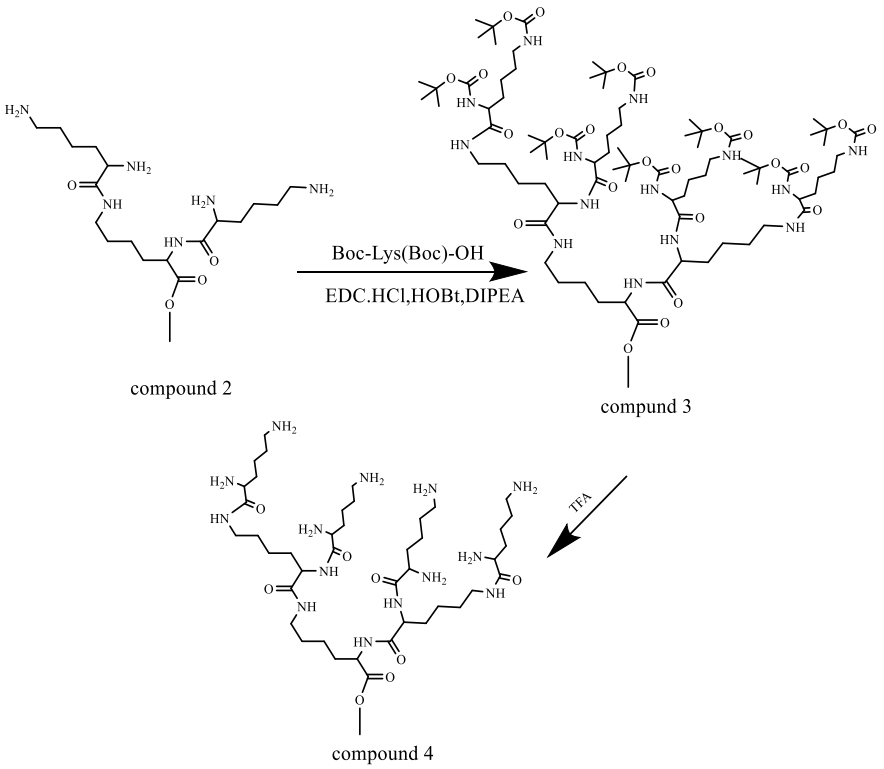
Synthesis of Generation 2 and 3 L-lysine dendrimers

Generations 2 and 3 of the L-lysine dendrimers were synthesized using the divergent method. Generations 2 and 3 were termed G2-Lys and G3-Lys. MeO-Lys-H (1.1 g, 1 equiv), Boc-Lys(Boc)-OH (6.2 g, 3 equiv), EDC (3.1 g, 3 equiv), HOBT (2.1 g, 3 equiv) and 40 mL DMF were added, and DIPEA (8.1 mL, 8 equiv) was dispersed in the DMF in an ice-bath. Reactants were stirred at 25 ℃for 24 h. DMF was then removed and the reactants were dissolved in chloroform. The reactants were washed with saturated NaCl, $NaHCO_3$ and HCl (~1 M). $MgSO_4$ was used as a desiccant to obtain raw products and silica columns (DCM/MeOH (20/1; v/v)) were used to purify the compound. Compound 1 was treated with TFA/DCM for 8 h to de-protect the Boc groups. Anhydrous diethyl ether was used to deposit compound 2. This process was repeated for G3-Lys. ^{1}H NMR spectra (400 MHz, Bruker Avance II NMR spectrometer, Germany) were used to characterize these compounds. Matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry (Bruker Autoflex III, Germany) was also used for compound analysis.

Scheme S1. G2-Lys synthesis

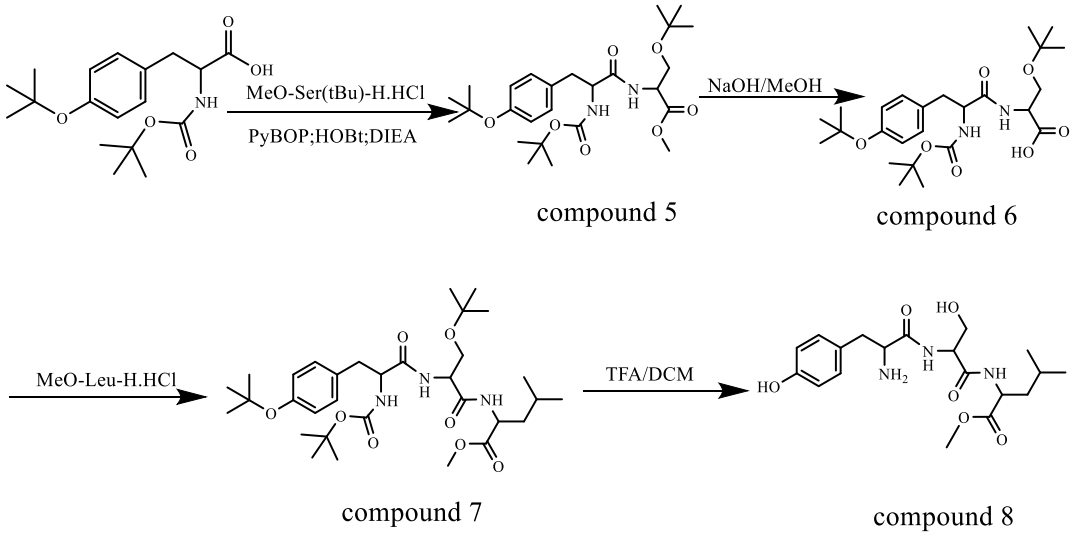


Scheme S2. G3-Lys synthesis



Synthesis of YSL

Scheme S3. YSL synthesis

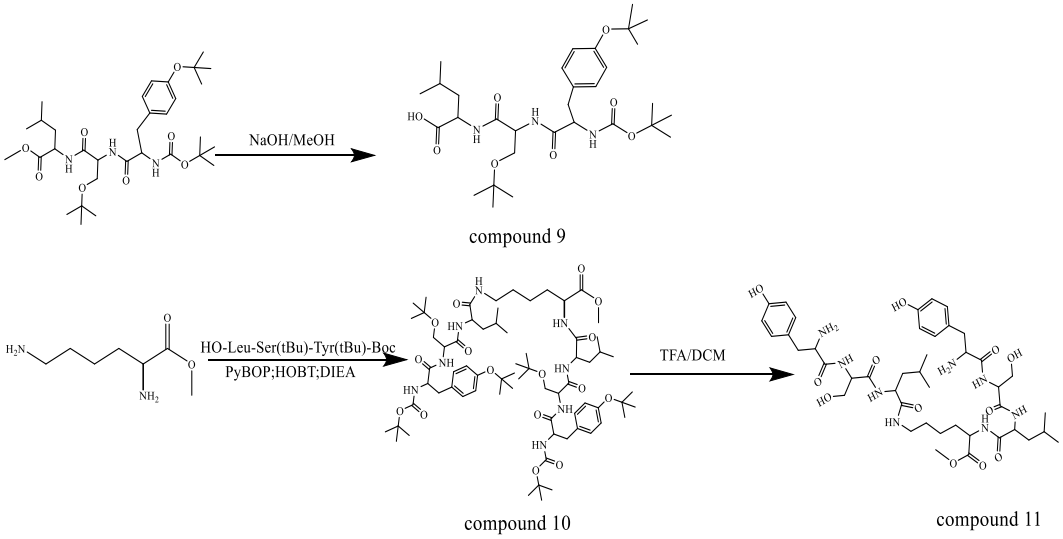


Boc-Tyr (tbu)-OH (3.0 g, 8.9 mmol), MeO-Ser (tBu)-H•HCl (1.8 g, 8.9 mmol), PyBOP (6.9 mg, 13.5 mmol), HOBT (1.4 mg, 13.5 mmol) were stirred in 35 mL anhydrous DMF in an N_2 atmosphere. DIPEA (8.8 mL, 53.4 mmol) was mixed at 0 °C After 36 h at room temperature, DMF was distilled and chloroform was added. Silica columns (PE/EA=5/1) were used for purification, yielding compound 5 (3.7 g, 86%). The methoxy group of compound 5 was deported using NaOH/MeOH (1 mol/L). Once the MeOH was removed, EA was used to dissolve the residue. HCl (1 mol/L) was used to adjust the pH to 2-3 so that compound 6 could be dissolved in EA. $MgSO_4$ was used to dry the solution, and the solvent was removed to obtain a white powder.

Compound 6 (2.5 g, 5.0 mmol), MeO-Leu-H•HCl (1.1 g, 5.0 mmol), PyBOP (3.8 g, 7.4 mmol) and HOBT (0.8 g, 7.5 mmol) were dissolved in DMF and DIPEA (5 mL, 30.3 mmol) and mixed under an N_2 atmosphere. The mixed liquor was stirred for 48 h at 25 °CThe post-treatments were the same as those used to obtain the raw product. The raw product was purified using silica columns (PE/EA=3/1) to obtain a white solid compound 7 (1.9 g, 62.7%). The Boc and tBu groups were then de-protected. Anhydrous diethyl ethers were used to treat the solution and a white solid YSL (yield 85%) product was obtained.

Synthesis of Bivalent YSL

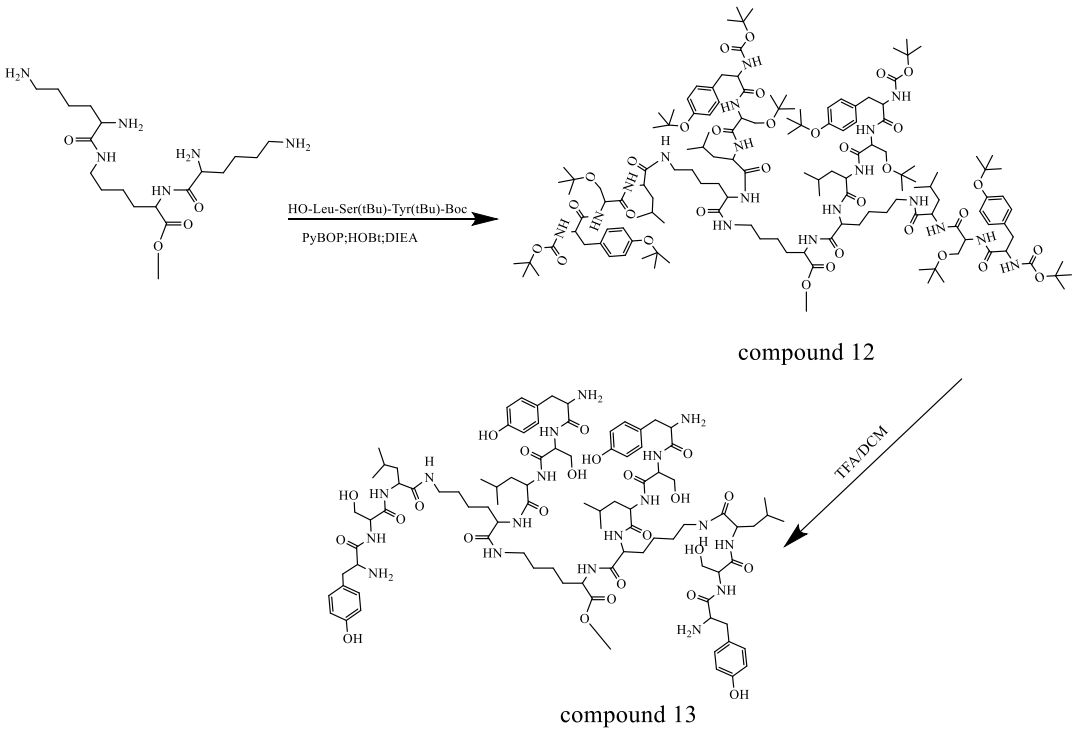
Scheme S4. Synthetic route of bivalent YSL



NaOH/MeOH (1 mol/L) was used to remove the methoxy group of compound 7. Once the MeOH was removed, EA was used to dissolve the residues. HCl (1 mol/L) was used to adjust the pH to 2~3 so that compound 6 could be dissolved in EA. Anhydrous $MgSO_4$ was used to dry the solution, and the solvent was removed. MeO-Lys-H (1.1 g, 4.2 mmol), compound 9 (6.2 g, 10.3 mmol), PyBOP (6.6 g, 12.8 mmol), HOBT (1.4 g, 12.8 mmol), 40 mL DMF and DIPEA (4.2 mL, 51.4 mmol) were added under an N_2 atmosphere. The post-treatments were identical to the raw production and purified on silica columns (PE/EA=1/1) to obtain a white solid compound 10 and the Boc and tBu groups were de-protected. The post-treatments were performed as described to obtain bivalent YSL (yield 70%).

Synthesis of Tetravalent YSL

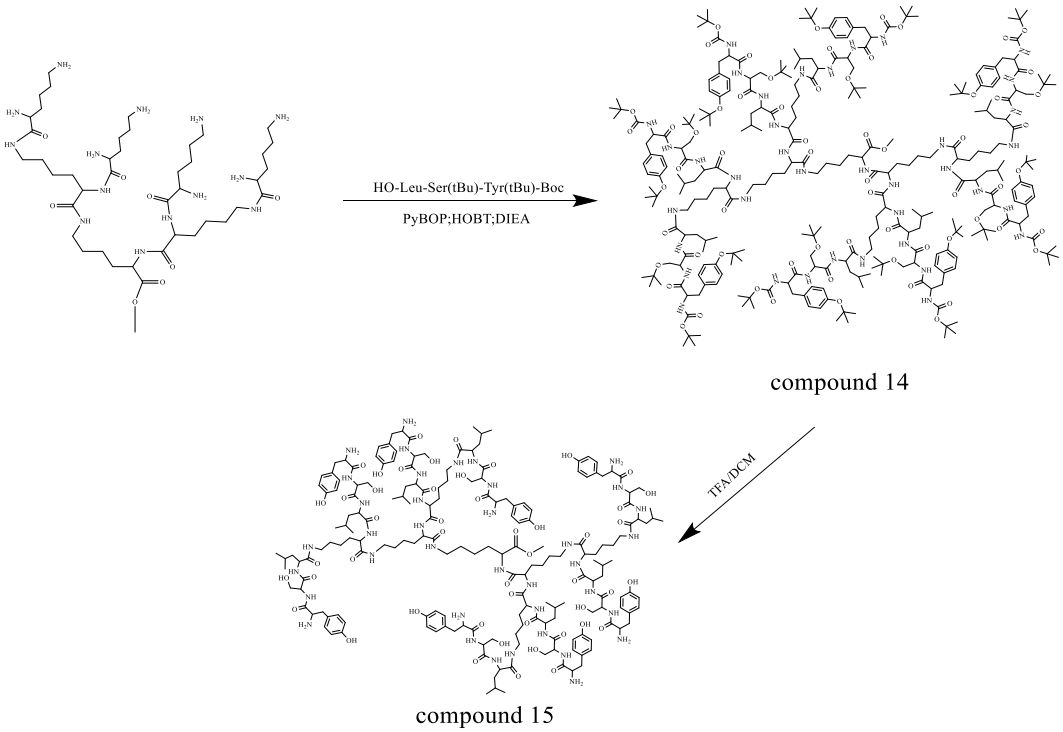
Scheme S5. Synthetic route of tetravalent YSL



Compound 2 (1.1 g, 1.2 mmol), compound 9 (5.9 g, 9.7 mmol), PyBOP (5.1 g, 9.7 mmol), HOBT (1.0 g, 9.7 mmol), DIPEA (4.8 mL, 29.2 mmol) and 40 mL DMF were added under an N_2 atmosphere. The mixed solution was reacted for 48 h and post-treatments were repeated to obtain the raw product. Anhydrous diethyl ether was used to treat the solution to obtain tetravalent YSL (yield 62%).

Synthesis of Octavalent YSL

Scheme S6. Route of octavalent YSL synthesis



Compound 4 (1.0 g, 0.5 mmol), compound 9 (5.0 g, 9.1 mmol), PyBOP (4.8 g, 9.1 mmol), HOBT (1.2 g, 9.1 mmol), DIPEA (4.5 mL, 27.3 mmol) and 40 mL DMF were added under an N_2 atmosphere. The mixture was reacted for 48 h at 25 ℃Post-treatments were the same as those described above. The purified product was obtained using silica columns (DCM/MEOH=10/1), and the Boc and tBu groups were de-protected. A white solid product in the form of octavalent YSL (yield 35%) was obtained.

HPLC

The purity of YSL, bivalent YSL, tetravalent YSL and octavalent YSL were detected via reversed-phase high-performance liquid chromatography (RP-HPLC) using a 1260-pump and a 1260-ultraviolet detector (Agilent, USA). An Agilent Zorbax Eclipse XDB-C18 column (150 × 4.6 mm, 5 μm) was used for chromatographic separation, linear gradient elution from A/B = 90/10 to A/B = 10/90 over 15 minutes using eluent A (0.1% TFA in water) and eluent B (0.1% TFA in CH_3CN), with a flow rate of 0.5 mL/min. UV detected at λ = 220 nm.

Circular Dichroism (CD) Spectra Generation

G1-Lys, G2-Lys, G3-Lys, YSL, bivalent YSL, tetravalent YSL and octavalent YSL were dissolved in water. Secondary structure composition was analyzed through the addition of the sample into a CD cuvette. A CD spectrometer (JASCO Corp, J-1500, Japan) was used to detect the CD spectra from 190 nm to 290 nm.

Resistance to Serum

Fetal Bovine Serum (FBS) were dissolved in saline (pH 7.4). Different generations of the multivalent YSL dendrimers were dissolved in saline (containing 10% FBS). After incubating at 37 ℃ for 0 h, 1 h, 2 h, 3 h, 4 h, 6 h and 12 h, approximately

150 μL of each miscible liquids was mixed with 50 μL of acetonitrile to inactivate the serum. The mixture was centrifuged at 8000 rpm for 5 min, supernatants were analyzed by RP-HPLC to monitor and quantify the degradation.

Cytotoxicity Testing

4T1 cells were used to assess the cytotoxicity of multivalent YSL dendrimers. Cytotoxicity was evaluated via MTT assay. Different concentrations of the indicated dendrimers were added to cells for 24 h (the YSL monomer molar concentrations of each compounds were same). Cells were then incubated in 100 μL of media containing 0.5 mg/mL MTT reagent for 2 h. The media was removed and DMSO was used to dissolve the resultant formazan product. A BioTek microplate reader was used to spectrophotometrically characterize the absorbance at 490 nm. Relative cell viability was then calculated: cell viability = (ODsample - ODbackground) / (ODcontrol - ODbackground) × 100%. The cytotoxicity of YSL, YSL+dendrimer (the YSL monomer molar concentrations of each mixture were same, and the lysine dendrimer scaffold molar concentrations of each mixture were same as the molar concentration of corresponding multimeric YSL ligand dendrimers), the Taxol against 4T1 cells was carried out similarly. We used the same method to analyze the cytotoxicity of multivalent YSL dendrimers on normal L929 cells.

Hemolysis

Fresh blood was obtained from BALB/c mouse. RBCs were washed with saline for 3 times using centrifugation at 1000 rpm for 5 min and diluted with saline solution (1:6). 500 μL diluted RBCs were mixed with 500 μL saline containing YSL, bivalent YSL, tetravalent YSL and octavalent YSL with different concentrations for 2 h (the YSL monomer molar concentrations of each compounds were same). Deionized water was used as a positive control and saline was used as a negative control. Next, the samples were centrifuged at 10000 rpm for 5 min. The supernatants were obtained and detected by a UV-Vis spectrophotometer (Specord 200, Analytik Jena, Germany) at 541 nm. The hemolysis percent of RBCs was calculated using the following equation: hemolysis percent = (Asample – Anegative) / (Apositive – Anegative) × 100%. The experiments were carried out in triplicate.

Live-dead cell staining

Live-dead kits were used to measure cell death using an inverted optical microscope (Leica DMI4000B, Germany). 4T1 cells were grown in 96-well microplates for 24 h, and multivalent YSL ligand dendrimers (the YSL monomer molar concentrations of each compounds were 0.4 mM or 0.8 mM) were then added for 24 h. Cells were stained using live dead kits based on provided directions.

Cell Cycle Distribution

4T1 cells were plated into 6-well plates and treated with 0.4 mM multivalent YSL ligand dendrimers (the YSL monomer molar concentrations of each compounds were 0.4 mM) or Taxol (10^{-3} mM) for 24 h. Cells were then collected via centrifugation and fixed in pre-cooled 70% ethanol. Cells were washed in PBS and stained with PI for 30 min. Flow cytometry was used to measure DNA content.

Annexin V-FITC/PI Staining

4T1 cells were plated into 6-well plates and treated for 24 h with 0.4 mM multivalent dendrimers (the YSL monomer molar concentrations of each compounds were 0.4 mM). Equal volumes of Taxol (10^{-3} mM) were used as a positive control for 24 h. Cells were washed three times in PBS and resuspended in 5 μL Annexin V-FITC and 5 μL PI for 20 min. Stained cells were collected and analyzed by FACS.

Wound Healing Assay

This assay was performed using 4T1 cells grown to confluence in 12-well plates, with a sterile 10 μL pipet tip being used to scratch the plate, generating a "wound" in the cell layer. After using PBS to wash this wound twice, cells were treated with the multivalent YSL dendrimers (the YSL monomer molar concentrations of each compounds were 0.2 mM). Alternatively, cells were treated with Taxol (10^{-5} mM) as positive control. The digitized images of the wound area were imaged with an inverted optical microscopy, and three representative fields of view were selected to calculate the average length of cell migration at 0 h, 24 h and 36 h. The migration ability of the cells was evaluated by measuring the area of the wounds. Each assay was performed in triplicate in three independent experiments.

Cell Migration Assay

4T1 cells (2×10^5) in RPMI-1640 medium were added to the upper chambers of a Transwell assay system (Corning, 0.8 μm membrane) in order to assess their capacity for migration. This media also contained the multivalent YSL dendrimers (the YSL monomer molar concentrations of each compounds were 0.2 mM). Alternatively, Taxol (10^{-5} mM) was used as positive control. RPMI-1640 containing 10% FBS was added to the lower chamber. After 24 h, the cells that migrated to the bottom of the membrane were stained with crystal violet solution for 15 min, and were then washed twice with PBS. The stained cells were counted under a microscope.

Cell Invasion Assay

In vitro invasion assays were conducted using Transwell chambers (6.5 mm, 8 μm pore size, Corning), coated with matrigel (Corning). Briefly, cells (2×10^5) in 200 μL serum-free RPMI-1640 containing YSL dendrimers (the YSL monomer molar concentrations of each compounds were 0.2 mM) Taxol (10^{-5} mM) were added into the upper chambers for 24 h, with 600 μL RPMI-1640 containing 10% serum added to the lower chamber. Cotton swabs were tehn used to wipe away cells that had not migrates across the membrane, while cells which had migrated to the lower surface of the filter were stained with 0.1% crystal violet for 15 min and counted using a light microscope in five randomly selected fields.

SEM Imaging of Tumor Cells

4T1 cells (1×10^5 cells per well) were cultured in 24-well plates for 24 h to allow for attachment. After that , culture media was removed and cells were incubated with YSL dendrimers (the YSL monomer molar concentrations of each compounds were 0.2 mM) or Taxol (10^{-5} mM) for 24 h. Cells were then washed thrice in PBS, followed by fixation with 2.5% glutaraldehyde for 30 min. An ethanol gradient (30, 40, 50, 70, 80, 85, 90, 95 and 100%) was used to dehydrate cells for 5 min per concentration, after which cells were coated with gold prior to SEM imaging.

Microtubule Staining

4T1 cells were seeded in confocal dish at a density of 8000 cells/well for 24 h to allow for attachment. After that, culture media was removed and cells were incubated with YSL dendrimers (the YSL monomer molar concentrations of each compounds were 0.2 mM) or Taxol (10^{-5} mM) for 24 h. Cells were then washed thrice in PBS and stained for 30 minutes with Alexa Fluor™ 488 phalloidin solution (6.6 μM) after fixation using 1% paraformaldehyde for 10min. Cell were then washed three more times in PBS, stained for 10 min with Hoechst 33342 (1 μg/mL), washed again, and imaged via CLSM.

Gelatin Zymography

4T1 cells (1×10^5 cells per well) were cultured in 12-well plates for 24 h to allow for attachment. These cels were then treated with 0.2 mM multivalent YSL ligand dendrimers (the YSL monomer molar concentrations of each compounds were 0.2 mM) in serum-free RPMI-1640 medium for 24 h, after which conditioned media from these cells was collected. Samples were then

separated via electrophoresis on 10% sodium dodecyl sulfate (SDS)/polyacrylamide gels containing enzyme substrate (0.1% gelatin for gelatin zymography). After electrophoresis, the gels were washed twice in washing buffer for 30 min at room temperature, and were then incubated in reaction buffer (10 mM at 37 °C for 48 h. Bands corresponding to activity were visualized by negative staining using Coomassie Brilliant blue R-250 (Bio-Rad Laboratories, Richmond, CA), and molecular weights were estimated by reference to prestained SDS-PAGE markers.

In Vivo Tumor Therapy

Chengdu Dashuo provided the BALB/c mice used in this study (6-8 weeks old, 20-22 g). Animal experiments were performed according to the guideliens of the ethics committee of Sichuan University. Mice were subcutaneously implanted with 4T1 cells (1×10^6/mouse). 4T1 tumor-bearing BALB/c mice were randomly divided into 6 groups (n=5/group). Mice were intratumorally injected with normal saline or the indicated multivalent YSL ligand dendrimers at days 0, 3, 6, 9, 12 and 15 post-implantation at a dose of 10 mg per kg of body weight (the YSL monomer molar concentrations of each compounds were same). Alternatively, mice were administered intratumoral Taxol (10 mg PTX/kg body weight) as a positive control. We monitored tumor volumes and weight every 3 days for 24 days. Following this observation period, mice were sacrificed, and solid tumors were collected and fixed in 4% formaldehyde to obtain hematoxylin and eosin (H&E) stained sections. Immunohistochemistry was also performed. Samples were fixed in 4% formaldehyde and H&E staining was used for histopathological analysis. Finally, sections were imaged on an inverted optical microscopy (Leica DMI4000B, Germany). Formula to calculate the tumor volume: $V\ [mm^3] = LW^2/2$; formula to measure the relative body weight: = (body weight) / (body weight when the treatment was initiated) × 100%.

Characterizations

Characterizations of multivalent YSL ligand dendrimers

Characterization of Generation 2 lysine dendrimer

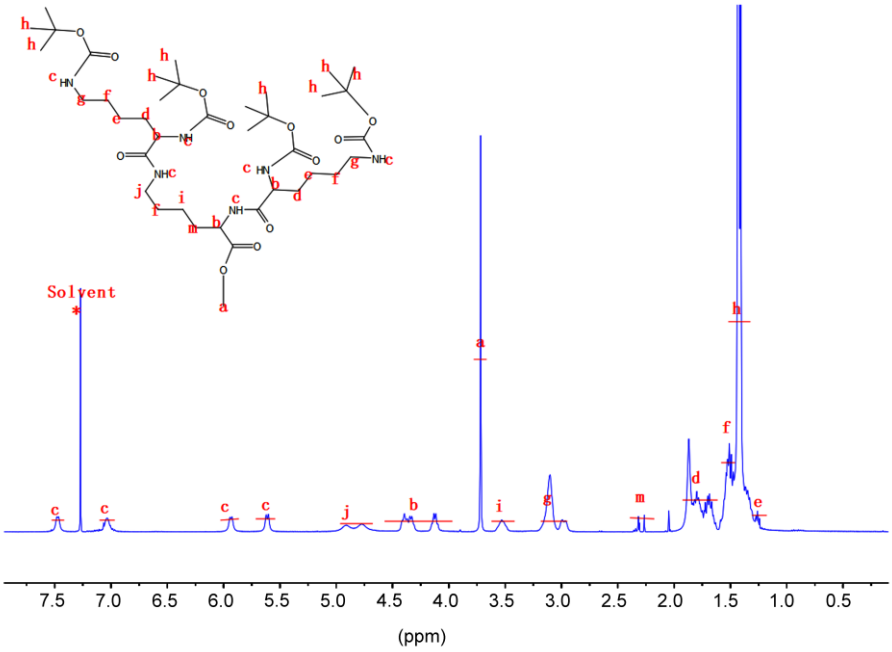


Figure S1. ^{1}H-NMR spectrum of protected G2-Lys in DMSO-d_6 (400 MHz)

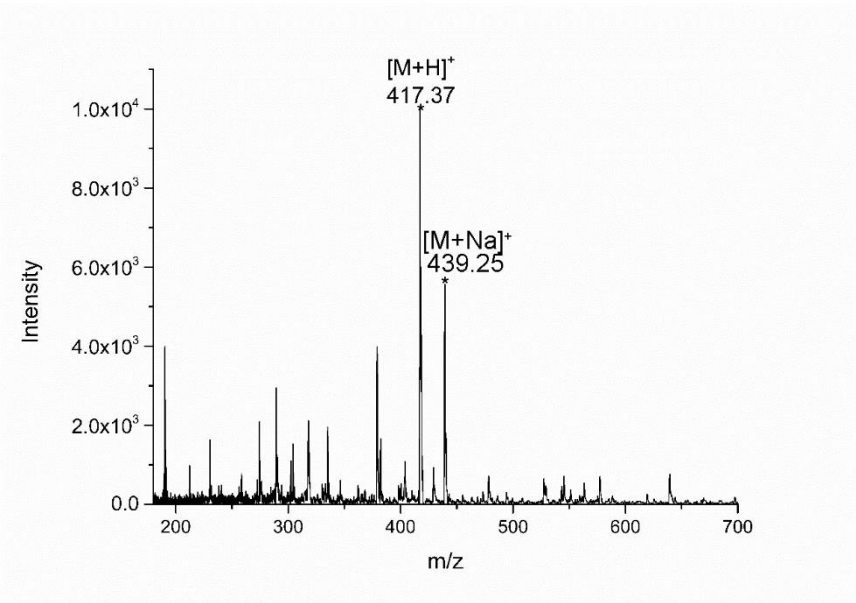


Figure S2. MALDI-TOF mass spectrum of G2-Lys (m/z, $[M+H]^+$):417.31 (calculated), 417.37 (observed), (m/z, $[M+Na]^+$):439.31 (calculated), 439.25 (observed).

Characterization of Generation 3 lysine dendrimer

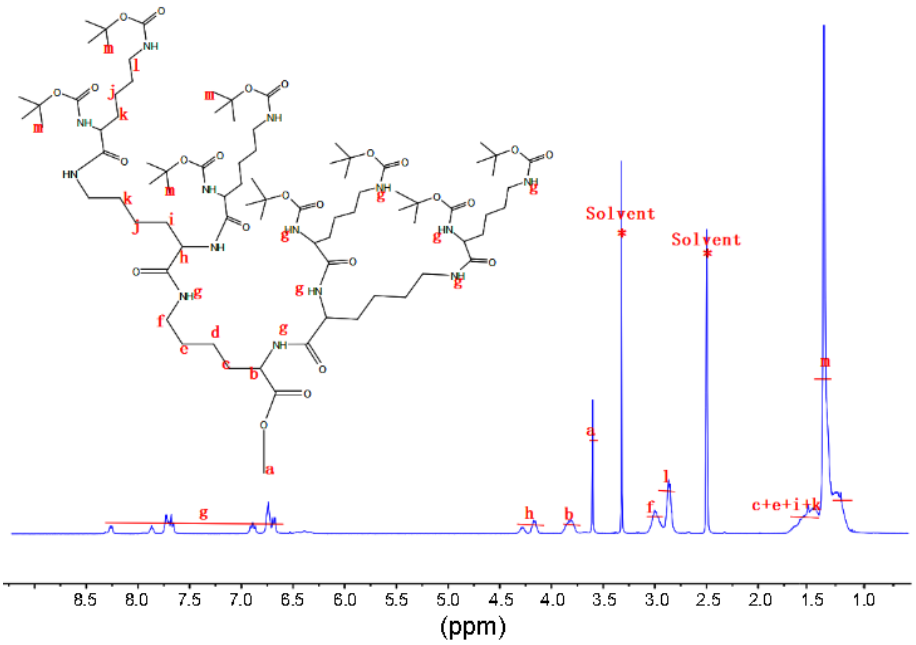


Figure S3. ^{1}H-NMR spectrum of protected G3-Lys in DMSO-d_6 (400 MHz)

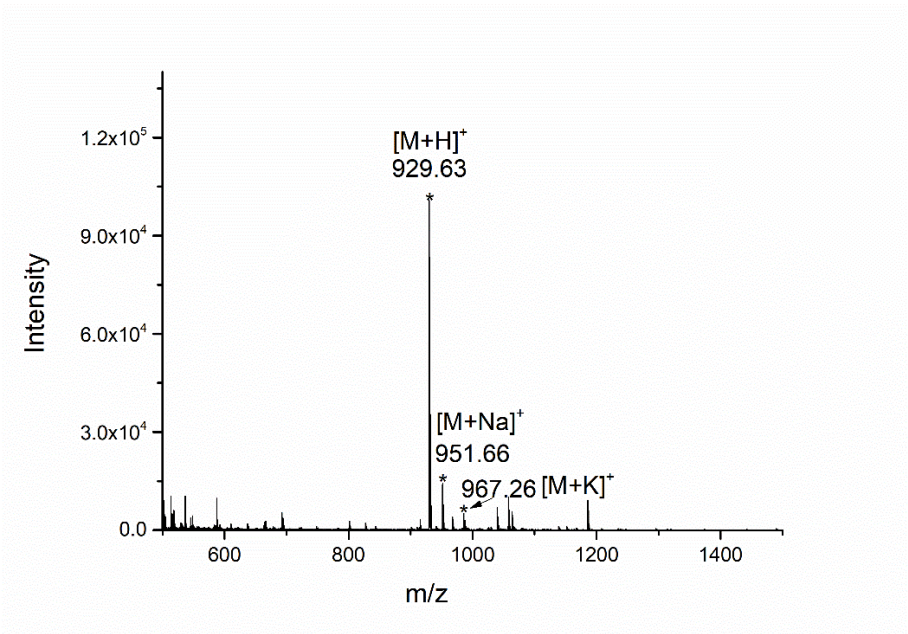


Figure S4. MALDI-TOF mass spectrum of G3-Lys (m/z, [M+H]$^+$): 929.69 (calculated), 929.63 (observed), (m/z, [M+Na]$^+$): 951.69 (calculated), 951.66 (observed), (m/z, [M+K]$^+$): 967.69 (calculated), 967.26 (observed).

Characterization of YSL

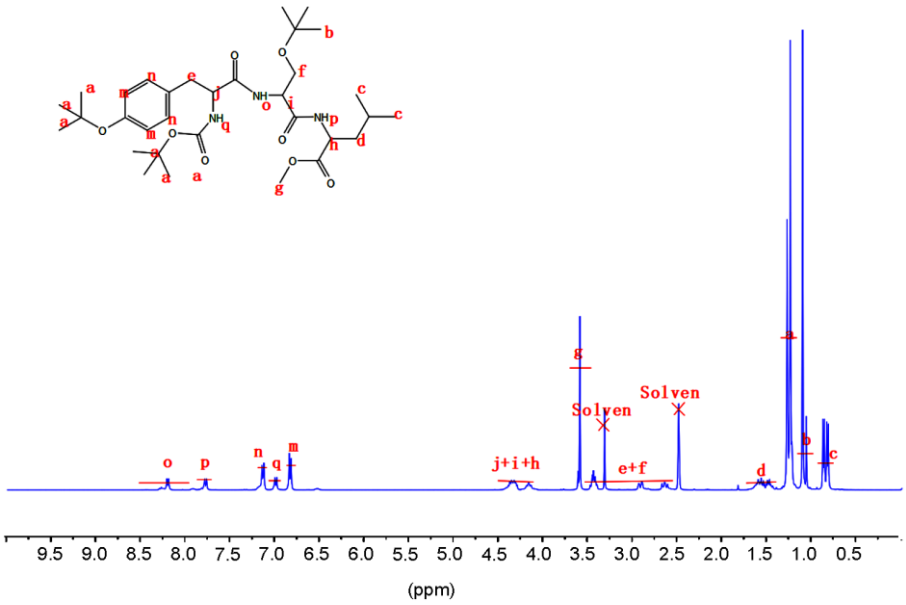


Figure S5. ^{1}H-NMR spectrum of protected YSL in DMSO-d_6 (400 MHz)

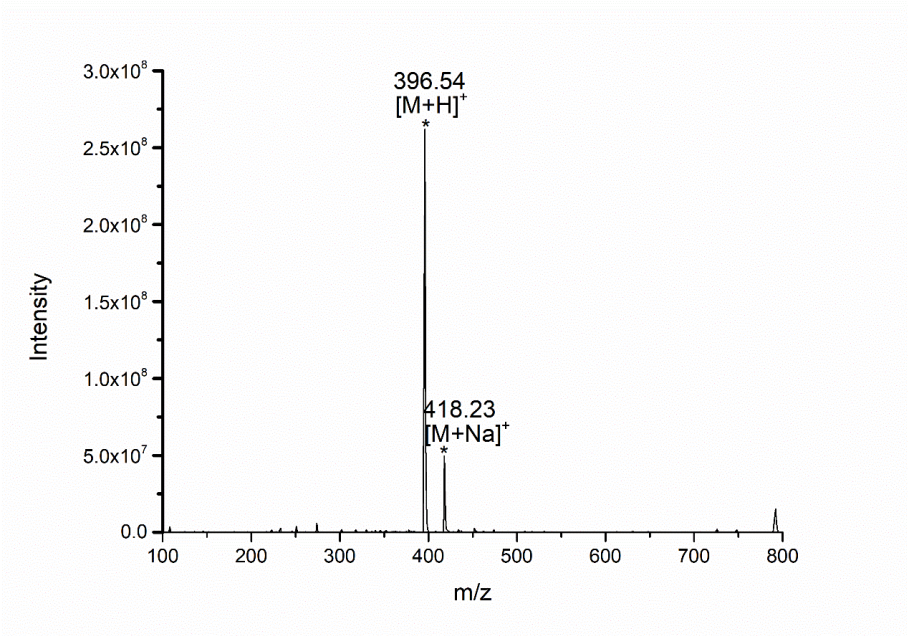


Figure S6. MALDI-TOF mass spectra of YSL (m/z, $[M + H]^+$): 396.21 (calculated), 396.54 (observed), (m/z, $[M + Na]^+$): 418.21 (calculated), 418.23 (observed).

Characterization of Bivalent YSL

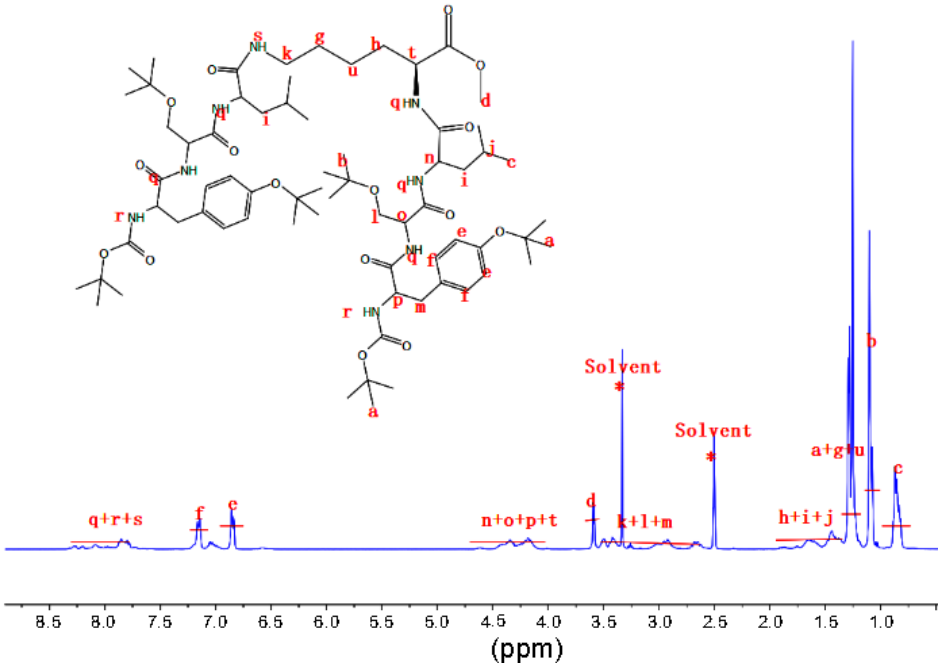


Figure S7. ^{1}H-NMR spectrum of protected bivalent YSL in DMSO-d_6 (400 MHz).

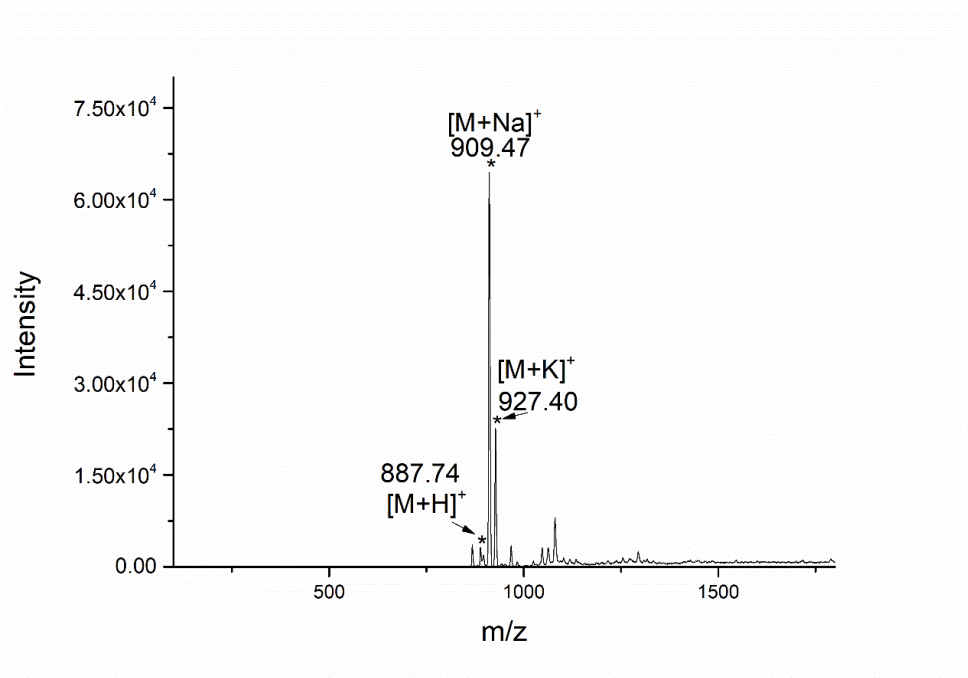


Figure S8. ESI mass spectra of bivalent YSL (m/z, [M + Na]$^{+}$): 909.41(calculated), 909.47 (observed), (m/z, [M + H]$^{+}$): 887.41(calculated), 887.74 (observed), (m/z, [M + K]$^{+}$): 927.41(calculated), 927.40 (observed).

Characterization of Tetravalent YSL

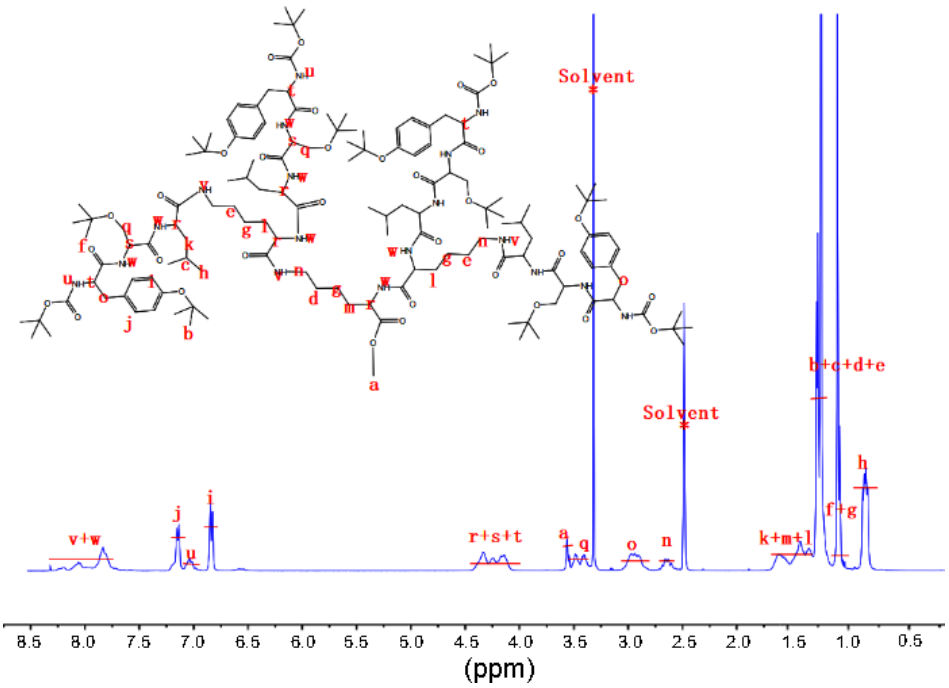


Figure S9. ^{1}H-NMR spectrum of protected tetravalent YSL in DMSO-d_6 (400 MHz).

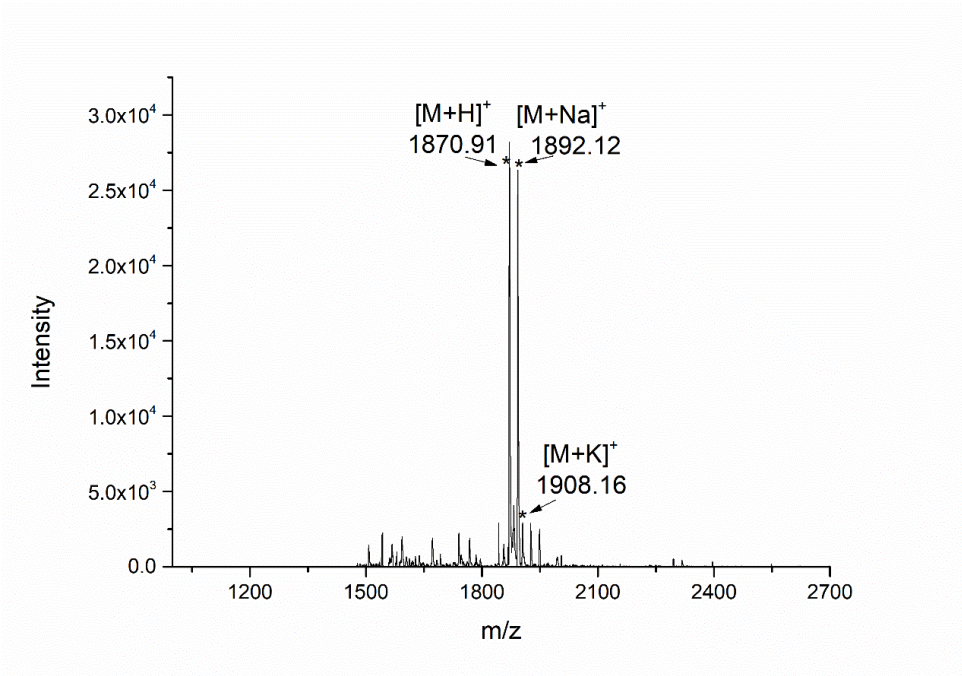


Figure S10. ESI mass spectra of of tetravalent YSL (m/z, $[M + H]^+$): 1870.27 (calculated), 1870.91(observed), (m/z, $[M + Na]^+$): 1892.27 (calculated), 1890.12 (observed), (m/z, $[M + K]^+$): 1908.27 (calculated), 1908.16 (observed).

Characterization of Octavalent YSL

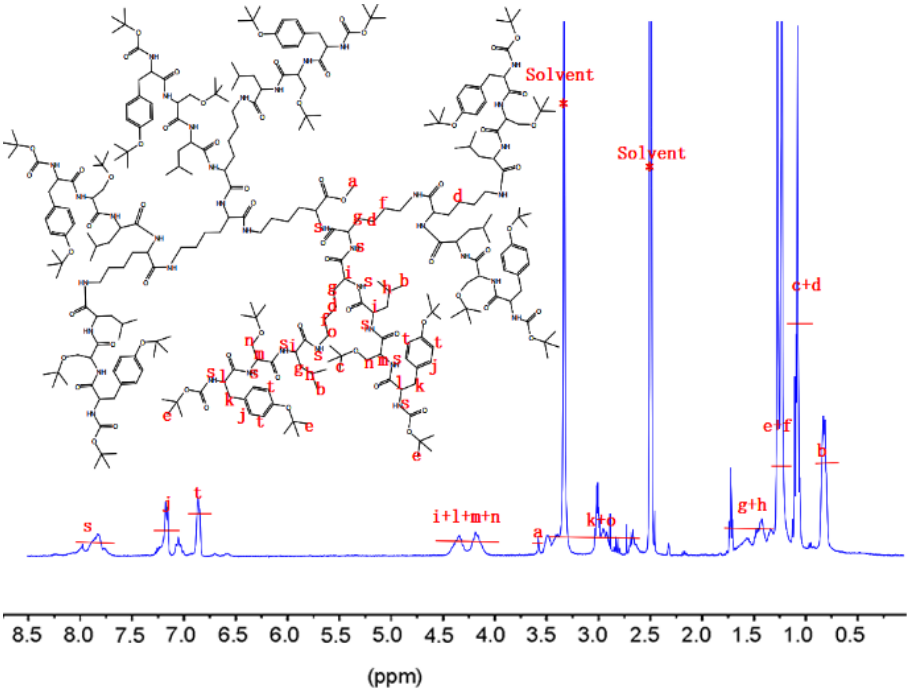


Figure S11. 1H-NMR spectrum of octavalent YSL in DMSO-d_6 (400 MHz).

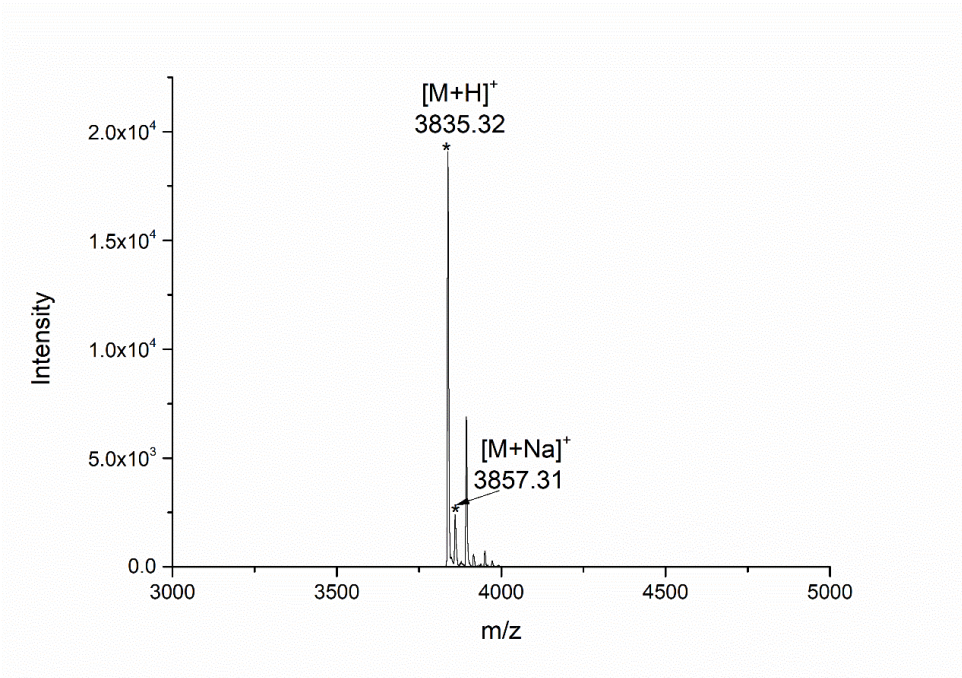


Figure S12. ESI mass spectra of octavalent YSL (m/z, $[M + H]^+$): 3835.12 (calculated), 3835.14 (observed), (m/z, $[M + Na]^+$): 3857.12 (calculated), 3857.31 (observed).

Table S1. Summary of Molecular Formula Parameters

Example	Formula	Adduct	Thero. m/z	Meas. m/z	Yield
YSL	$C_{19}H_{29}N_3O_6$	M+H	396.21	396.54	85%
Bivalent YSL	$C_{43}H_{66}N_8O_{12}$	M+Na	909.41	909.47	70%
Tetravalent YSL	$C_{91}H_{140}N_{18}O_{24}$	M+H	1870.27	1870.91	62%
Octavalent YSL	$C_{187}H_{288}N_{38}O_{48}$	M+H	3835.12	3835.32	35%

HPLC Analysis

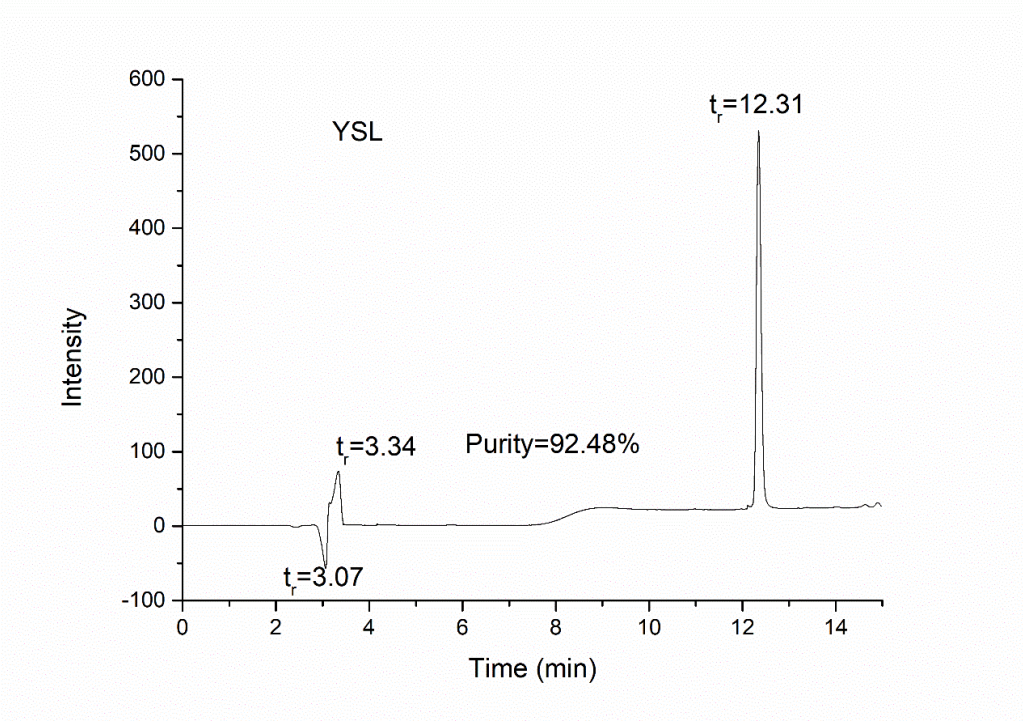


Figure S13. HPLC analysis of YSL. Gradient: A/B = 90/10 to A/B = 10/90 in 15 min; A (0.1% TFA in water); B (0.1% TFA in CH_3CN); flow rate: 0.5 mL/min; detection: λ=220 nm.

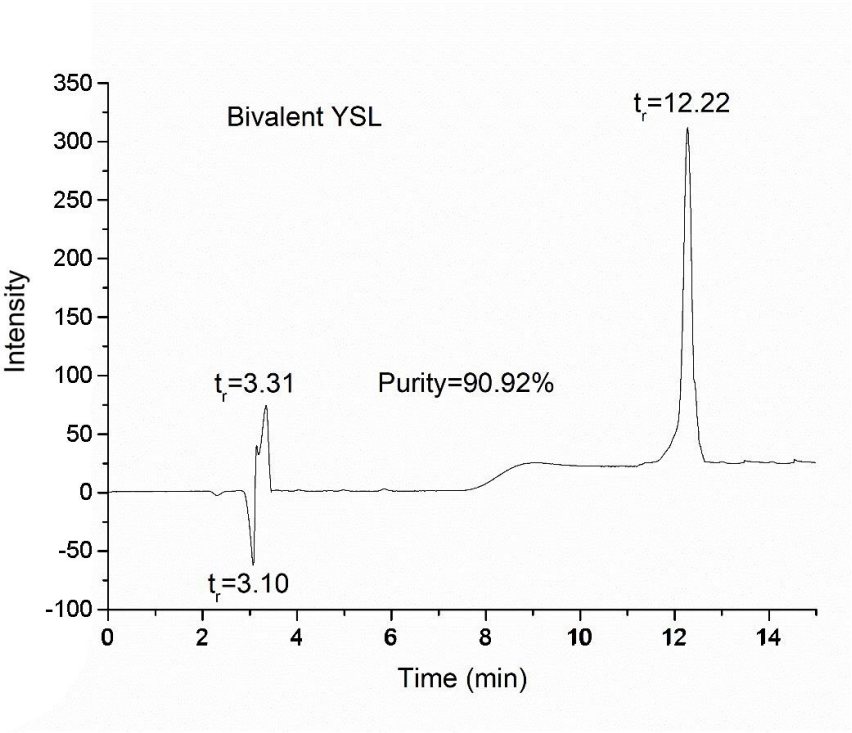


Figure S14. HPLC analysis of bivalent YSL. Gradient: A/B = 90/10 to A/B = 10/90 in 15 min; A (0.1% TFA in water); B (0.1% TFA in CH_3CN); flow rate: 0.5 mL/min; detection: λ=220 nm.

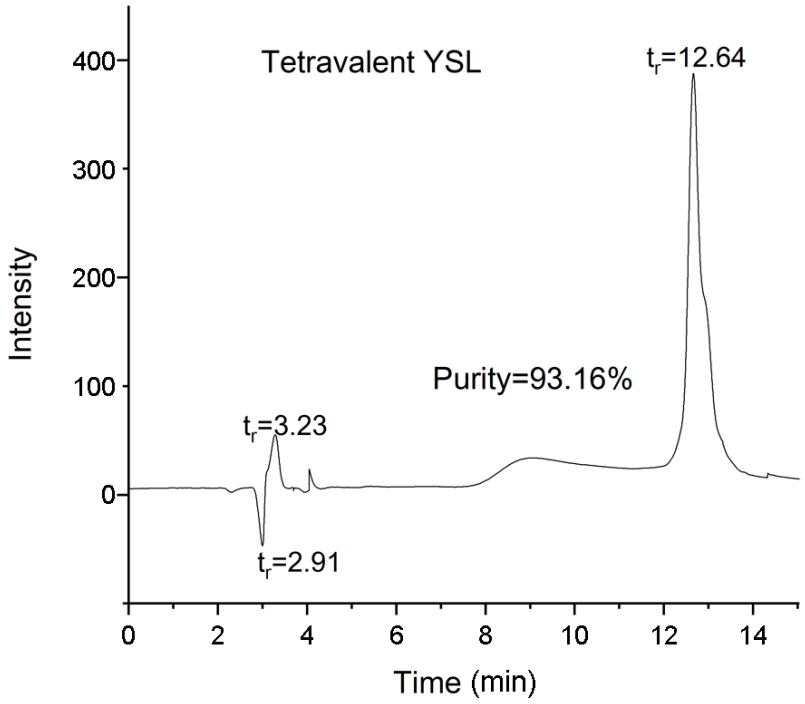


Figure S15. HPLC analysis of tetravalent YSL. Gradient: A/B = 90/10 to A/B = 10/90 in 15 min; A (0.1% TFA in water); B (0.1% TFA in CH_3CN); flow rate: 0.5 mL/min; detection: λ=220 nm

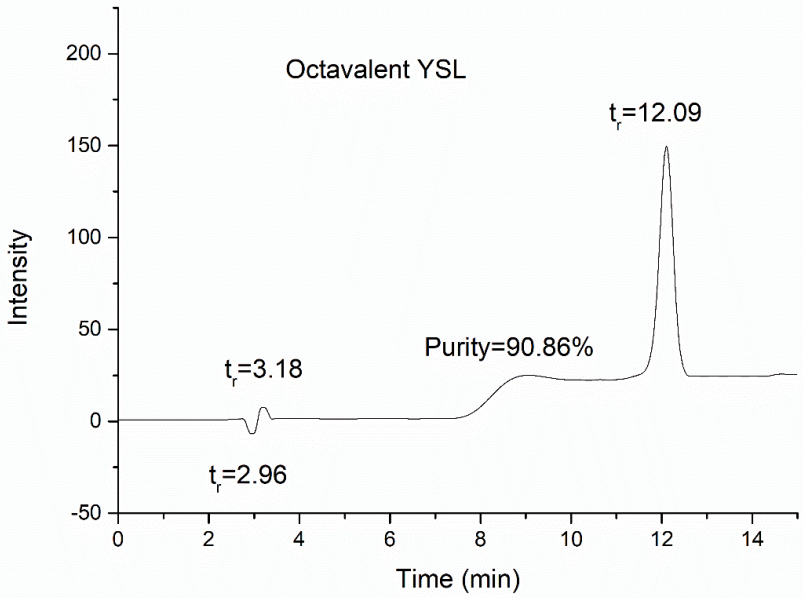


Figure S16. HPLC analysis of octavalent YSL. Gradient: A/B = 90/10 to A/B = 10/90 in 15 min; A (0.1% TFA in water); B (0.1% TFA in CH_3CN); flow rate: 0.5 mL/min; detection: λ=220 nm

Circular Dichroism Spectra

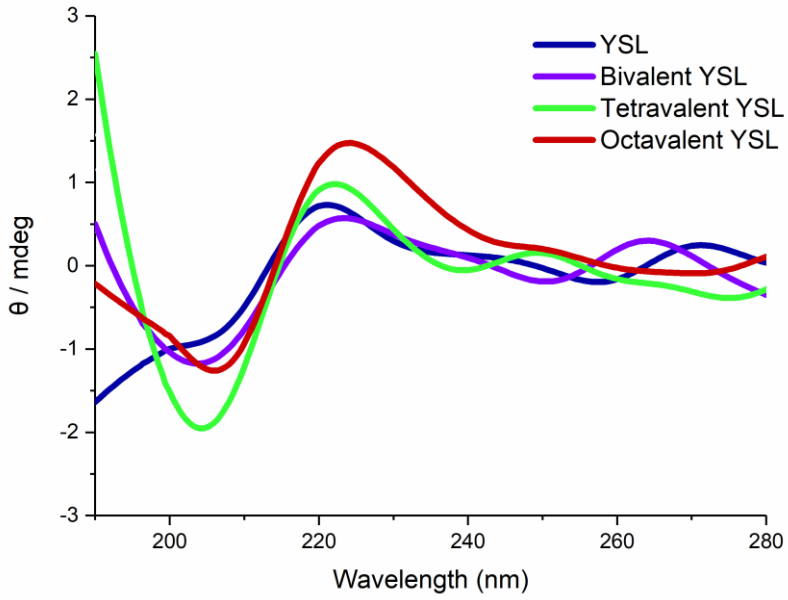


Figure S17. CD spectra of YSL-dendrimers.

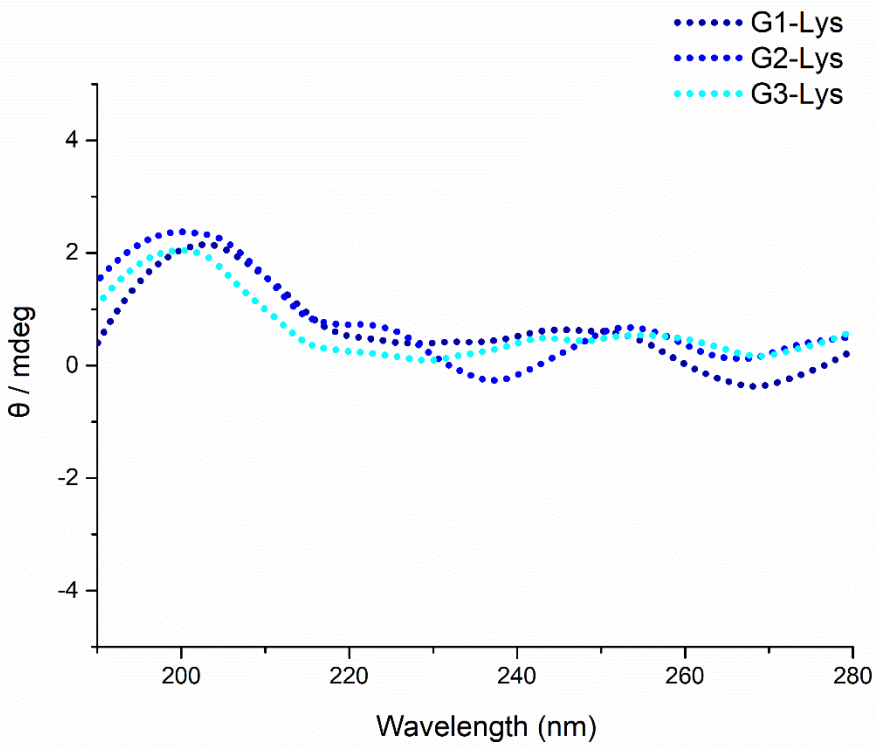


Figure S18. CD spectra of G1-Lys, G2-Lys, and G3-Lys.

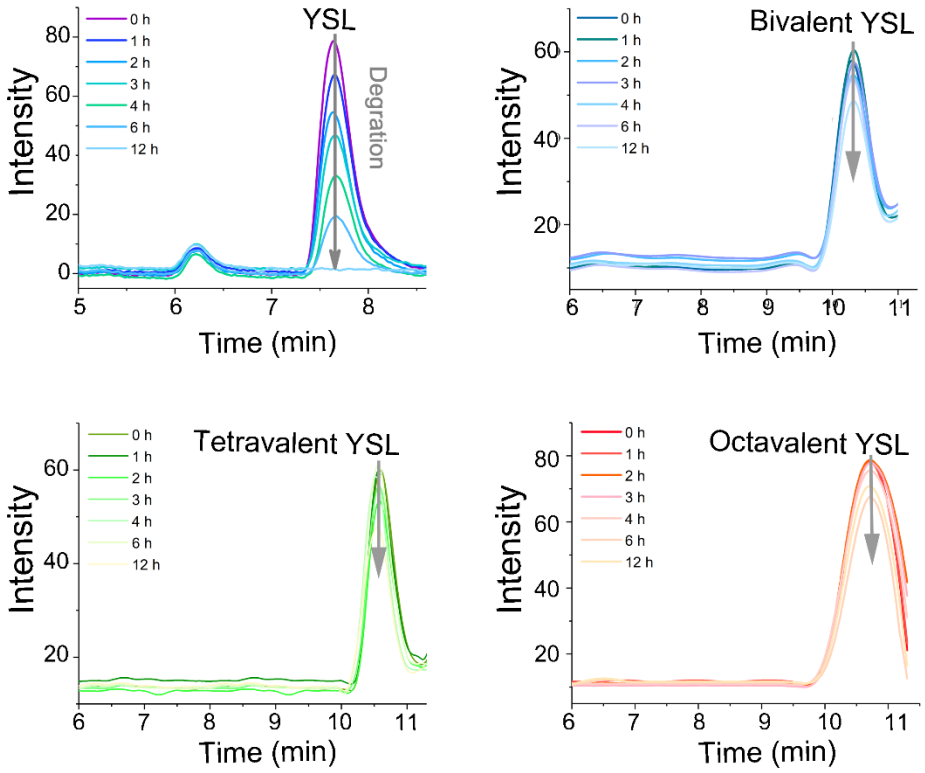


Figure S19. HPLC results of YSL-dendrimers after co-incubation with serum for different incubation times.

Cytotoxicity Testing

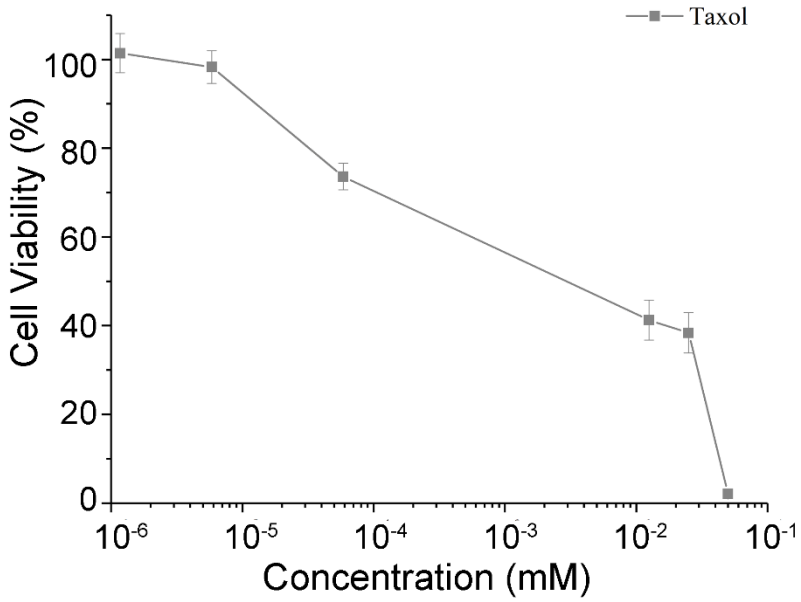


Figure S20. Viability of 4T1 cells treated with different concentrations of Taxol for 24 h (mean ± SD, *n* =6).

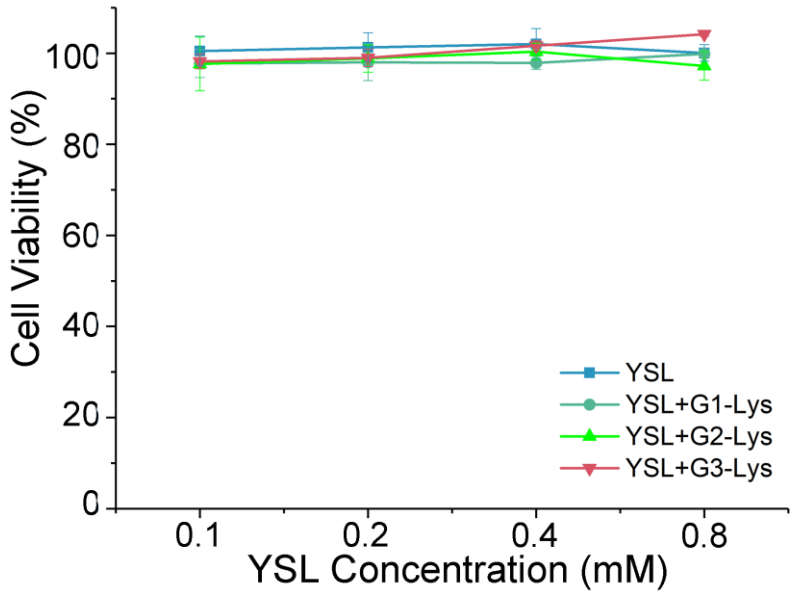


Figure S21. Viability of 4T1 cells after treatment with the homogenous bivalent, tetravalent and octavalent polylysine dendrimers mixed with YSL for 24 h (mean ± SD, *n* =6).

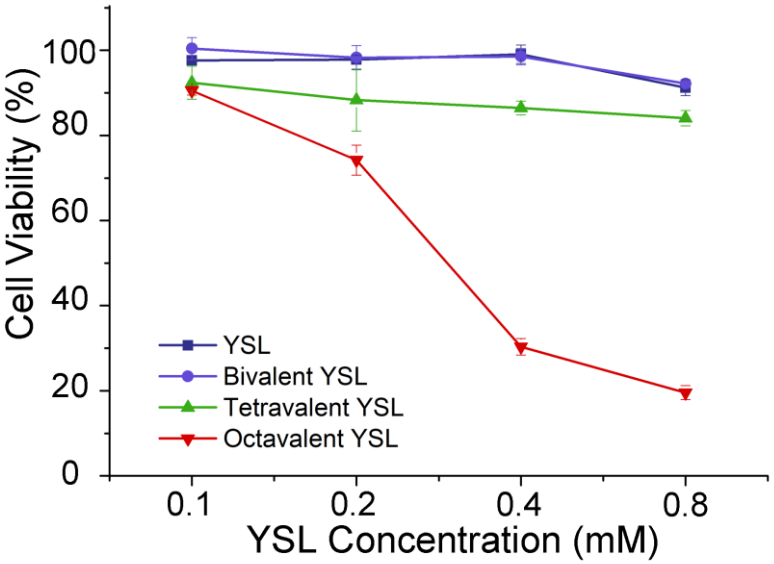


Figure S22. Cell viability of L929 cells after a 24 h incubation with different concentrations of YSL-dendrimers (mean ± SD, *n* =6.).

Hemolytic properties on mice erythrocytes.

Figure S23. Photographs of mouse RBC pellets after treated with water, saline, YSL and YSL-dendrimers (the concentration of YSL was 0.8 mM).

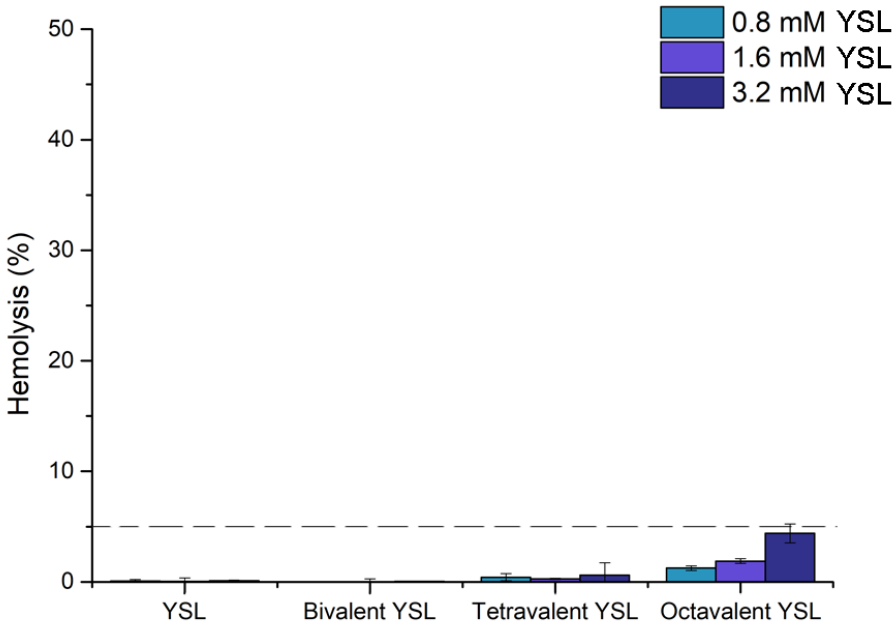


Figure S24. Hemolysis percentage of mouse RBCs after treated with different concentrations of YSL-dendrimers (mean ± SD, n =3, the YSL monomer molar concentrations of each compounds were same).

Apoptosis assessments via FDA/PI Staining

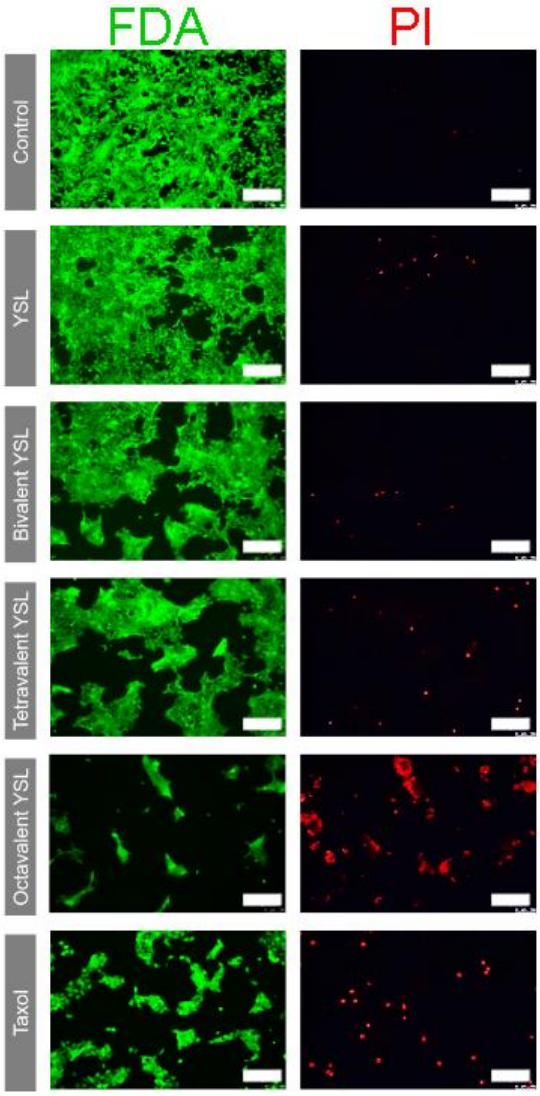


Figure S25. Microscopy images of 4T1 cells after treatment with multivalent YSL dendrimers (the concentration of YSL was 0.8 mM) for 24 h with FDA/PI staining. Scale bar: 200 μm. Cells positive for PI staining were classified as apoptotic.

Wound-Healing Assays

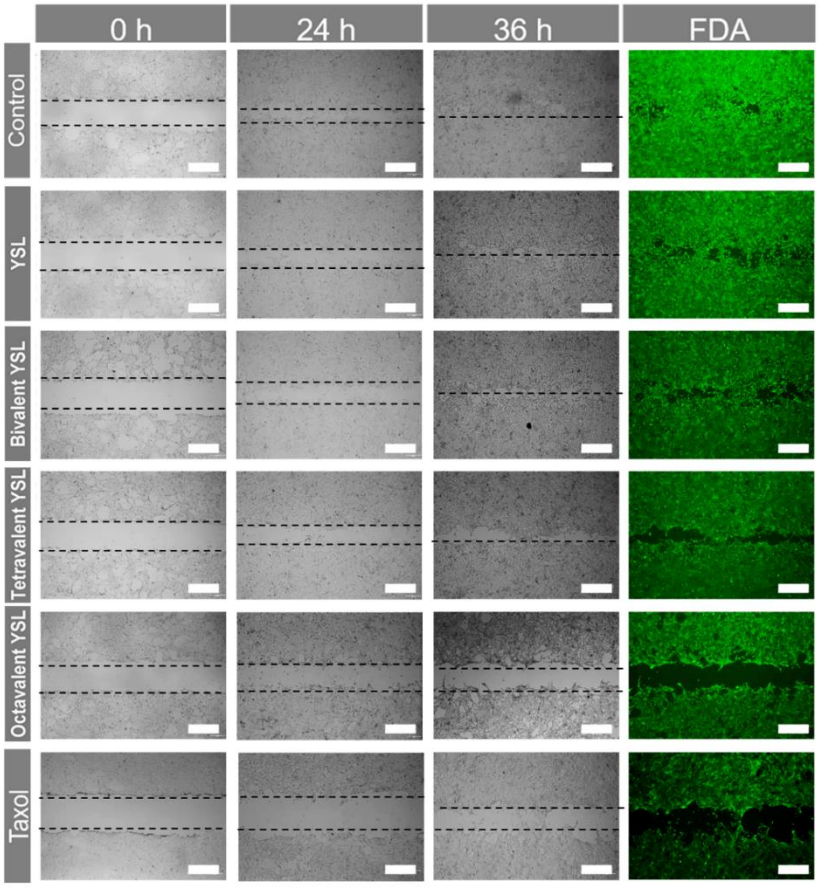


Figure S26. In vitro scratch wound healing assays. Multivalent YSL dendrimers (the concentration of YSL was 0.2 mM) inhibited the migration of 4T1 cells in vitro after 24 h or 36 h of treatment. Scale bar: 400 μm.

Gelatin zymography Assays

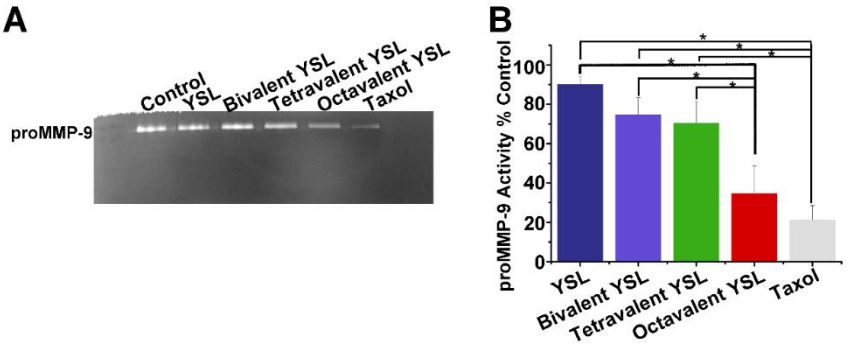


Figure S27. (A) Gelatin zymography analysis of supernatants from untreated, multivalent YSL dendrimer-treated, and Taxol-treated 4T1 cells after 24h. (B) MMP-9 activity was quantified based upon densitometry (means ± SD, n = 3, *$p \leq 0.05$).

In Vivo Antitumor Efficacy

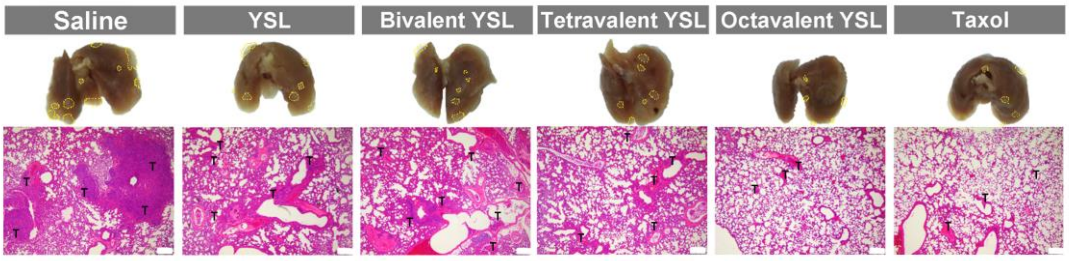


Figure S28. Representative whole lung photographs and images of H&E stained lung sections (T indicates the lung metastasis). Scale bar: 250 μm.

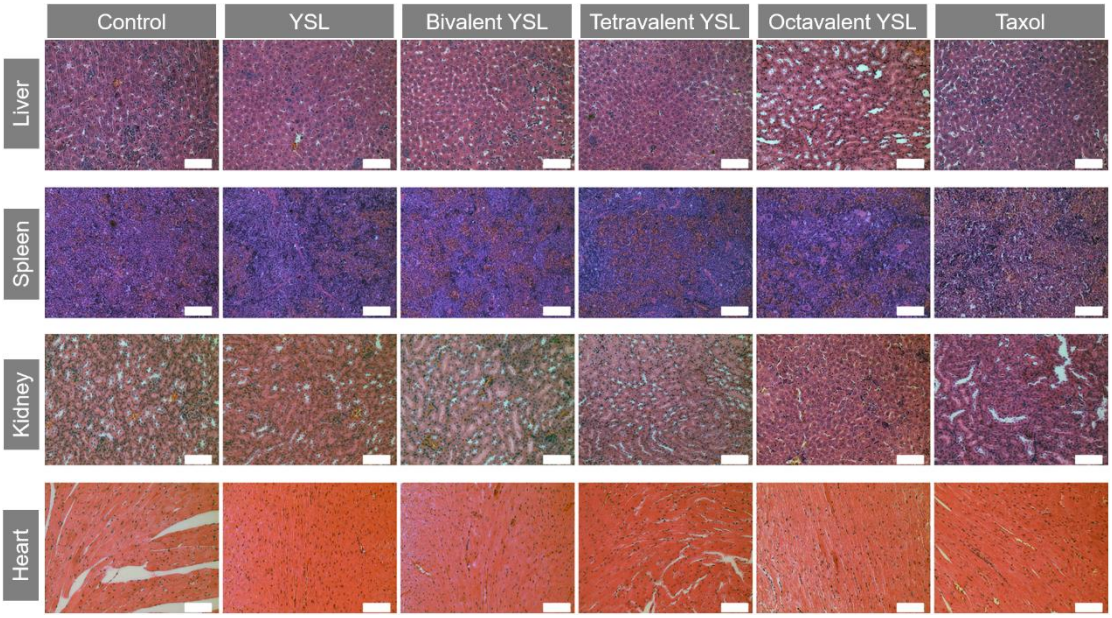


Figure S29. Histological and immunohistochemical images of hematoxylin and eosin (H&E) stained organ including the liver, spleen, kidney and heart after treatment for 24 days. Scale bar: 100 μm.

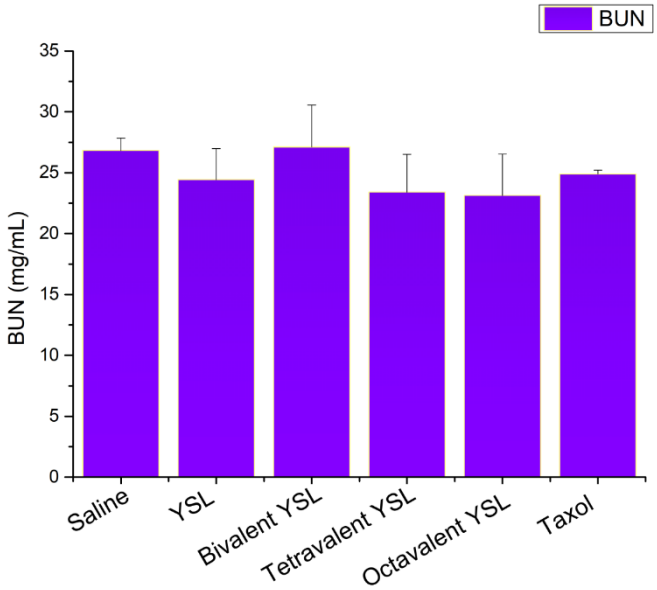


Figure S30. BUN levels of mice in different groups.

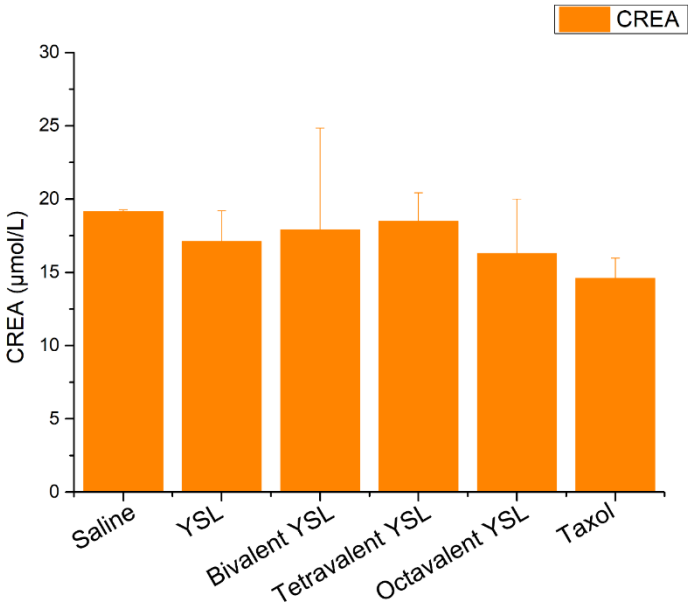


Figure S31. CREA levels of mice in different groups.