Electronic Supplementary Material (ESI) for Polymer Chemistry.

Fabrication of Thermoresponsive Nanogels by Thermo-Nanoprecipitation and in situ Encapsulation of Bioactives

Michael Giulbudagian‡, Mazdak Asadian-Birjand‡, Dirk Steinhilber, Katharina Achazi, Maria Molina, and Marcelo Calderón*

Freie Universität Berlin, Institute for Chemistry and Biochemistry, Takustr. 3, 14195 Berlin, Germany

Helmholtz Virtuelles Institut – Multifunctional Biomaterials for Medicine, Helmholtz-Zentrum Geesthacht, Teltow, Germany

SUPPORTING INFORMATION

Materials

Commercially available chemicals have been used as delivered. Solvents were purchased as reagent grade and distilled if necessary. Anhydrous solvents were either purchased as ultra-dry solvent from Acros Organics® or received from solvent purification system. Glycidyl methyl ether (GME) (85%, TCI Europe, Eschborn, Germany) and ethyl glycidyl ether (EGE) (98%, TCI Europe) were dried over CaH_2, distilled, and stored over molecular sieves (5 Å). For the polymerization reactions, dry toluene was obtained from MBRAUN SPS 800 solvent purification system. Water was purified by Millipore water purification system. Dendritic polyglycerol (dPG) with average Mw of 10 kDa (PDI = 1.3) was synthesized according to previously reported methodologies.[1] The amine functionalization of dPG in different loadings was performed as reported by Haag et al.[2] The synthesis of the cyclooctyne carbonate linker (1R,8S,9s)-Bicyclo[6.1.0]non-4-yn-9-ylmethyl (4-nitrophenyl) carbonate was performed as reported elsewhere.[3] The synthesis of linear thermo responsive polymers was performed according to a slightly modified procedure reported by Carlotti et al.[4]

Methods

Gas Permeation Chromatography (GPC)

Analytical GPC measurements were performed on an Agilent 1100 Series instrument including a UV detector (254 nm) as well as a refractive index detector. PS standards have been used for calibration and calculation was performed with PSS Win-GPC software. The measurements were run in THF as the eluent (1 mL min^{-1}, 20 °C), using an array of Suprema Lux 100, Suprema 1000, and Suprema Lux 3000 columns (dimensions: 8 x 300 mm, particle size: 10 μm, PSS, Mainz, Germany).

NMR Spectroscopy

The following spectrometers were used for recording ^{1}H NMR and ^{13}C NMR spectra: Bruker ARX 300 (300 MHz spectra), Bruker DRX 400 (400 MHz spectra), Bruker DRX 500 (500 MHz spectra), AMX 500 (500 MHz spectra) for the diffusion-ordered NMR (DOSY) experiments a Bruker AVANCE III 700 (700 MHz spectra) was used. Typically 10-30 mg of compound was used for recording ^{1}H NMR while 50-100 mg of compound was required for ^{13}C NMR. Deuterated solvents were used as standardized procedure. All spectra were recorded at r.t. and were analyzed with MestReNova software.

IR spectroscopy

FT-IR analysis was carried out using a JASCO FT-IR 4100 LE spectrophotometer in the range of 4000–500 cm^{-1}.

Dialysis

Benzoylated regenerated cellulose membrane purchased from Sigma-Aldrich, 2 kDa MWCO, and regenerated cellulose membrane purchased from SpctraPor, 50 kDa MWCO was used to perform dialysis. Typically dialysis was carried out for 24 h with 1 L of solvent that was exchanged after first 6 h of the process.

Size Exclusion Chromatography (SEC) and Thin Layer Chromatography (TLC)

Size exclusion chromatography (SEC) was performed with Sephadex G 25 Fine from GE Healthcare. The material was activated by swelling in the respective eluent prior to performing

chromatography. TLC was performed on Merck aluminum sheets with silica (corn size 60) and fluorescence marker (F254).

Dynamic light scattering (DLS)

Size, size distribution, and thermoresponsive behavior of tNGs were measured at various temperatures ranging from 15 to 80 °C by dynamic light scattering using a Nano-ZS 90 Malvern equipped with a He–Ne laser (λ = 633 nm) under scattering of 173°. All the samples were maintained for stabilization at the designed temperature for 5 min before testing. The samples were prepared dissolving 5 mg of dry nanogel in 1 mL of buffer phosphate pH = 7.4 one day prior to the experiments. Particle sizes and size distribution are given as the average of 3 measurements from the intensity distribution curves.

Nanoparticle tracking analysis (NTA)

Size, concentration of particles, and approximate molecular weight of the tNGs were measured dy NTA using a Nanosight NS500. The samples were prepared by diluting 1000 times the solution prepared for DLS measurements. Particle sizes, concentration, and molecular weight are given as the average of 3 measurements.

Atomic force microscopy (AFM)

AFM measurements were recorded by a tapping mode with a MultiMode 8 AFM equipped with a Nanoscope V controller from Veeco Instruments, Santa Barbara, California. The data were analyzed using NanoScope Analysis 1.3 software and statistical analysis were performed in a 5 µm x 5 µm image. The tNGs aqueous solutions (2 mg mL^{-1}) were spin coated on a Mica sheet at 90 rps for 5 min. Samples were analyzed by Nano World tips, Non-Contact/Tapping Mode-Long Cantilever (NCL-W), with resonance frequency of 190 kHz and force constant of 48 N m^{-1}.

Transmission electron microscopy (TEM)

A droplet of the sample solution (5µL) was applied on a hydrophilised (60 s glow discharging at 8 W using a BALTEC MED 020 device) collodium-supported carbon-coated copper grid (400 mesh, Plano, Wetzlar) for 60 s. The supernatant fluid was removed by blotting with a filter paper; then a droplet (5 µl) of 1% (w/v) uranyl acetate has been applied for another 60 s. The contrasting material was removed by means of filter paper and the sample was allowed to dry in the air.

TEM measurements were carried out at room temperature using a Philips CM12 (FEI Company, Oregon, USA) instrument equipped with a LaB6 cathode operated at 100 kV accelerating voltage. Exposures were made at the low-dose mode (< 100 e/Å) at a primary magnification of 58,300´.

Cloud point determination by UV-Visible measurement

Cloud points were measured on a Cary 100 Bio UV-Vis spectrophotometer equipped with a temperature-controlled, six-position sample holder. Buffer phosphate pH 7.4 nanogels solutions (5 mg mL^{-1}) were heated at 0.2 °C min^{-1} while monitoring both the transmittance at 500 nm (1 cm path length) and the solution temperature (from 15 to 80 °C), as determined by the internal temperature probe. The cloud point (CP) of each nanogel was defined as the temperature at the inflection point of the normalized transmittance curves.

Encapsulation of Doxorubicin HCl

Similar to the described synthetic procedure of tNG (tNG08), tPG-azide (7 mg, 1.2 µmol, 4.2 µmol azide) and Dox (11 mg, 19 µmol) were dissolved in 0.5 mL DMF and 5 drops of dimethyl sulfoxide (DMSO) and mixed with a solution of dPG-Oct (4 mg, 0.4 µmol, 5.6 µmol cyclooctynes) dissolved in 0.5 mL DMF. The mixture was cooled down in an ice bath, shaken for one minute and injected into the non-solvent (20 mL H_2O, 45 °C). A uniform dispersion was formed upon the injection of the polymers. The formed particles were let to react for 16 h followed by quenching with azidopropanol (20 µmol). After additional 3 h the mixture was cooled down and concentrated under reduced pressure. Following, the mixture was transferred into a centrifugal filter device (Amicon Ultra-4, 3K) and washed 4 times with water. The amount of the encapsulated Dox was estimated by UV-Vis spectroscopy (λ = 488 nm, ε = 11,500 L mol^{-1} cm^{-1}). The loading weight percentage was calculated by the following equation, where *Wdox* and *Wng* stand for the encapsulated Dox weight and the weight of the tNG respectively:

$$Loading\ wt.\ \% = \frac{Wdox}{Wdox + Wng} \times 100\%$$

Cellular studies

Cytotoxicity

The toxicity of the nanogels in tumor cells was investigated by MTT assay and real time cell analysis (RTCA). Briefly, A549 cells (adenocarcinomic human alveolar basal epithelial cells)

cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (BioChrom KG, Berlin, Germany), 100 U mL^{-1} penicillin, and 100 µg mL^{-1} streptomycin at 37 °C, 5% CO_2, and 99% humidity, were seeded in a 96-well (10.000 cells per well). After approximately 18 h the nanogel tNG09, Table S1) was added in serial dilutions. PBS and doxorubicin treated cells served as controls. 72 h post treatment, cell culture medium was removed an 10 µL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent (0.5 mg mL^{-1} in PBS) in 100 µL cell culture medium was added. After 2 h of incubation the MTT containing medium was replaced by 100 µL DMSO to dissolve the formazan crystals and absorbance was measured at 570 nm. For the RTCA measurement a 96-well E-plate was used instead of a simple 96-well plate. For the real time measurement the E-plate was placed in an RTCA SP device (Roche, Mannheim, Germany) inside an incubator (5% CO_2 and 99% humidity). Impedance was measured at least every 15 min. For analysis of end point data GraphPad Prism 5.01 software was used.

Confocal laser scanning microscopy (cLSM)

Cellular uptake of Cy5-conjugated nanogels (tNG10) was monitored by confocal microscopy. For the uptake study 50.000 A549 cells were seeded on 9 mm glass coverslips in each well of a 24-well plate and cultured for 24 h before adding the nanogel for 18 h. Cells were grown at 37 °C and 5% CO_2 and maintained in Dulbecco's minimal essential medium (DMEM with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin. For qualitative analysis by confocal laser scanning microscopy, cells were washed 3 times with PBS and fixed with 4% paraformaldehyde for 20 min. Afterwards, cells were permeabilized with 0.1% TritonX for 5 min and washed 2 times with PBS. To detect co-localization with early endosomes, samples were incubated 1 h with FITC labeled Early Endosome Antibody EEA1 (2.5 µg mL^{-1}) in the dark at 37 °C and rinsed 3 times with PBS. Cell nuclei and were stained with 4',6-diamidino-2-phenylindole (DAPI). Cells were observed and imaged using a confocal laser scan microscope (Leica DMI6000CSB stand).

Synthetic Methodologies

Synthesis of linear thermoresponsive polyglycerol (tPG)

Synthesis of tPG, 1:1 GME/EGE ratio will be described as a general procedure for polymerization reactions. The reactions proceed under argon atmosphere in a reaction flask which was heated under vacuum prior introduction of the reagents. $NOct_4Br$ (tPG_{5kDa}: 208 mg, 0.38 mmol; tPG_{15kDa} : 69 mg, 0.13 mmol) was added into a flask equipped with a magnetic stirrer, and dried under vacuum at 70

°C. Toluene (18 mL) was added into the flask followed by the addition of GME (0.88 g, 10 mmol) and EGE (1.02 g, 10 mmol). The reaction mixture was cooled down to 0 °C, avoiding the precipitation of the ammonium salt. The polymerization was activated by the addition of *i*-Bu_3Al solution in toluene (tPG_{5kDa}: c = 1 M, 1.44 mL, 1.54 mmol; tPG_{15kDa}: c = 1 M, 0.48 mL, 0.51 mmol) and let to proceed for 16 h at r.t. The reaction was quenched by the addition of ethanol and purified by dialysis (2 kDa molecular weight cut-off [MWCO] membrane) in toluene for 72 h. Yield : tPG_{5kDa} 1.67 g (83 %); tPG_{15kDa} 1.84 g (92 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 3.89-3.94 (m, 1H, terminal O<u>H</u>), 3.39-3.71 (m, 409 H, polymer backbone), 3.33 (s, 105 H, -OC<u>H</u>$_3$), 1.16 (t, 82 H, -OCH_2C<u>H</u>$_3$). FT-IR: ν (cm^{-1}) = 2870, 1456, 1198, 1103, 961, 930, 872. GPC: (tPG_{5kDa}) M_n = 4746 g mol^{-1}, M_w = 5175 g mol^{-1}, M_z = 5586 g mol^{-1}, PDI = 1.09, (tPG_{15kDa}) M_n = 13,839 g mol^{-1}, M_w = 17,055 g mol^{-1}, M_z = 20,065 g mol^{-1}, PDI = 1.67.

Azidation of tPG

The previously synthesized linear polyglycerol, tPG_{5kDa} (1 g, 0.2 mmol) and tPG_{15kDa} (1 g, 60 µmol) were dried in a 50 mL flask at 110 °C under vacuum and dissolved in 7 mL of dry tetrahydrofuran (THF). Followed by the addition of triethyl amine (TEA) (tPG_{5kDa}: 404 mg, 4 mmol; tPG_{15kDa}: 121 mg, 1.2 mmol) the reaction mixture was placed in an ice bath and methan sulfonyl chloride (tPG_{5kDa}: 229 mg, 2 mmol; tPG_{15kDa}: 69 mg, 0,6 mmol) was added dropwise via a syringe. The reaction was stirred at room temperature for 16 h. The ammonium salt was removed by filtration and the reaction mixture was purified by dialysis (benzoylated cellulose dialysis tubes, Sigma-Aldrich, 2 kDa MWCO) against methanol for 1 day. The solvent was removed by reduced pressure and a yellow, honey like product was obtained. Yield: tPG_{5kDa}-Ms 856 mg (86 %); tPG_{15kDa}-Ms 747 mg (75 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 3.39-3.71 (m, 409 H, polymer backbone), 3.33 (s, 105 H, -OCH_3), 3.09 (m, 3H, -SO_3-CH_3), 1.16 (t, 82 H, -OCH_2CH_3). FT-IR: ν (cm^{-1}) = 2871, 1456, 1198, 1108, 963, 930.

The mesylated tPG (tPG_{5kDa}-Ms: 800 mg, 0.16 mmol; tPG_{15kDa}-Ms: 700 mg, 0.046 mmol) was dissolved in 7 mL of dry DMF. NaN_3 (tPG_{5kDa}-Ms: 208 mg, 3.2 mmol; tPG_{15kDa}-Ms: 60 mg, 0.92 mmol) was added and the suspension was stirred at 60 °C for 3 days according to published procedures for the azidation of dPG.[5] The brownish salt was filtered and the reaction mixture was purified by dialysis (benzoylated cellulose dialysis tubes, Sigma-Aldrich, 2 kDa MWCO) against methanol for 2 days. The solvent was removed by reduced pressure to obtain a yellow, honey like product. Yield: tPG_{5kDa}-azide 608 mg (76 %); tPG_{15kDa}-azide 581 mg (82 %). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 3.39-3.71 (m, 409 H, polymer backbone), 3.33 (s, 105 H, -OCH_3), 1.16 (t, 82 H, -OCH_2CH_3). FT-IR: ν (cm^{-1}) = 2871, 2099, 1456, 1379, 1198, 1103, 961, 882.

Synthesis of dPG cyclooctyne carbamate (dPG-Oct)– a: 5%, b: 10%, and c: 20%

Previously synthesized dPG amine (a: 70 mg, 47 µmol NH_2-groups, 1 eq., b: 65 mg, 88 µmol NH_2-groups, 1 eq., c: 40 mg, 108 µmol NH_2-groups, 1 eq.) was dissolved in 3 mL of DMF. Followed by the addition of TEA (a: 14 mg, 0.018 mL, 141 µmol, 3 eq.; b: 27 mg, 0.037 mL, 264 µmol, 3 eq.; c: 33 mg, 0.045 mL, 324 µmol, 3 eq.) the reaction mixture was placed in an ice bath and (1R, 8S, 9s)-bicyclo[6.1.0]non-4-yn-9-ylmethyl (4-nitrophenyl) carbonate (a: 18 mg, 56 µmol, 1.2 eq.; b: 33 mg, 106 µmol, 1.2 eq.; c: 41 mg, 129 µmol, 1.2 eq.) was added to the reaction mixture. Formation of a yellow color was observed. After stirring for 1 h at r.t., the product was purified by dialysis (benzoylated cellulose dialysis tubes, Sigma-Aldrich, 2 kDa MWCO) against methanol for 1 day. A 1 mL aliquote was taken out of the purified product solution and dried on a rotation evaporator at 30 °C. The dried aliquote was further used for characterization via NMR and for determining the reaction yield. Yield dPG-Oct a: 77 mg (99%); b: 82 mg (99%); c: 60 mg (99%). ^{1}H NMR (400 MHz, CD_3OD): δ (ppm) = 3.41-4.15 (m, 2H, dPG backbone), 2.04-2.40 (m, 6H, cyclooctyne), 1.28-1.38 (m, 2H, cyclooctyne), 0.64-0.89 (m, 3H, cyclopropane). FT-IR: ν (cm^{-1}) = 3375, 2918, 2361, 1697, 1540, 1456, 1256, 1111, 853.

Synthesis of thermoresponsive nanogels (tNGs) via Thermo-Nanoprecipitation (TNP)

In a typical synthesis, as for tNG, a solution of tPG-azide (6 mg, 1.2 µmol, 4.2 µmol azide) in 0.5 mL DMF was mixed with a solution of dPG-Oct (0.5 mg, 50 nmol, 0.35 µmol octynes) dissolved in 0.5 mL DMF in an ice bath. The mixture was shaken for one minute and injected into the non-solvent (20 mL H_2O, 45 °C). A uniform dispersion was formed upon the injection of the polymers. The formed particles were let to react for 16 h. Following, the unreacted cyclootynes were quenched with 3-azidopropanol (40 µmol) or alternatively (Table S1, tNG10) with azide functionalized Cy5 dye (10 µmol). After additional 3 h the mixture was cooled down and the product was purified by dialysis against water (50 kDa MWCO, regenerated cellulose membrane). A 1 mL aliquote was taken out of the purified product solution and dried on a rotation evaporator at 30 °C. The dried aliquote was further used for characterization via NMR and for determining the reaction yield. tNG-Cy5 conjugate formation was confirmed by chromatography on thin layer chromatography (methanol/water, v/v 1:1), and appearance of a band on a Sephadex G 25 column. The synthesized tNGs and the reaction conditions are summarized in Table S1. Yield 70-95%. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) = 3.237-3.726 (m, dPG and tPG backbone), 3.20 (s, -OC$\underline{H}_3$ tPG), 1.06 (t, -$OCH_2C\underline{H}_3$ tPG). FT-IR: ν (cm^{-1}) = 2975, 2868, 1642, 1455, 1379, 1306, 1197, 1106, 961, 865.

Table S1 Nanogel batches and physico-chemical properties.

Batch	tPG $\bar{M}w$ (kDa)[a]	dPG-Oct functional groups[b]	dPG-Oct wt. %[c]	Total macromonomer conc. (mg mL^{-1})[d]	tNG Diameter (nm), PDI[e]
tNG01	5	7	8	6.5	295, 0.26
tNG02	5	14	16	7	181, 0.20
tNG03	5	28	16	7	188, 0.21
tNG04	5	14	41	2	154, 0.23
tNG05	5	14	41	4	126, 0.12
tNG06	5	14	41	5	122, 0.12
tNG07	5	14	41	8	180, 0.07
tNG08	5	14	41	11	91, 0.06
tNG09	5	14	41	24	331, 0.29
tNG10	15	14	41	8.5	104, 0.09
tNG11	15	28	8	6.5	125, 0.12
tNG12	15	28	16	7	176, 0.15

[a] Determined by GPC. [b] Determined by ^{1}H NMR. [c] Weight percentage of the total feed weight. [d] Concentration of the macromonomers in the solvent (DMF). [e] Polydispersity index (PDI) determined by DLS at 25 °C, average of 3 measurements from the intensity distribution curves.

1D-^{1}H-DOSY-NMR (700 MHz, D_2O): Data were fit using the following equation:

I = I[0]*exp(-D*SQR(2*PI*gamma*Gi*LD)*(BD-LD/3)*1e4) where:

I[0] = 4.664e-01

D= Diff Con. = 3.070e-12 m^2/s

Gi= Gamma = 4.258e+03 Hz/G

LD= Little Delta = 4.000 m

BD= Big Delta = 200.000 m

RSS = 4.987e-05

SD = 6.242e-04

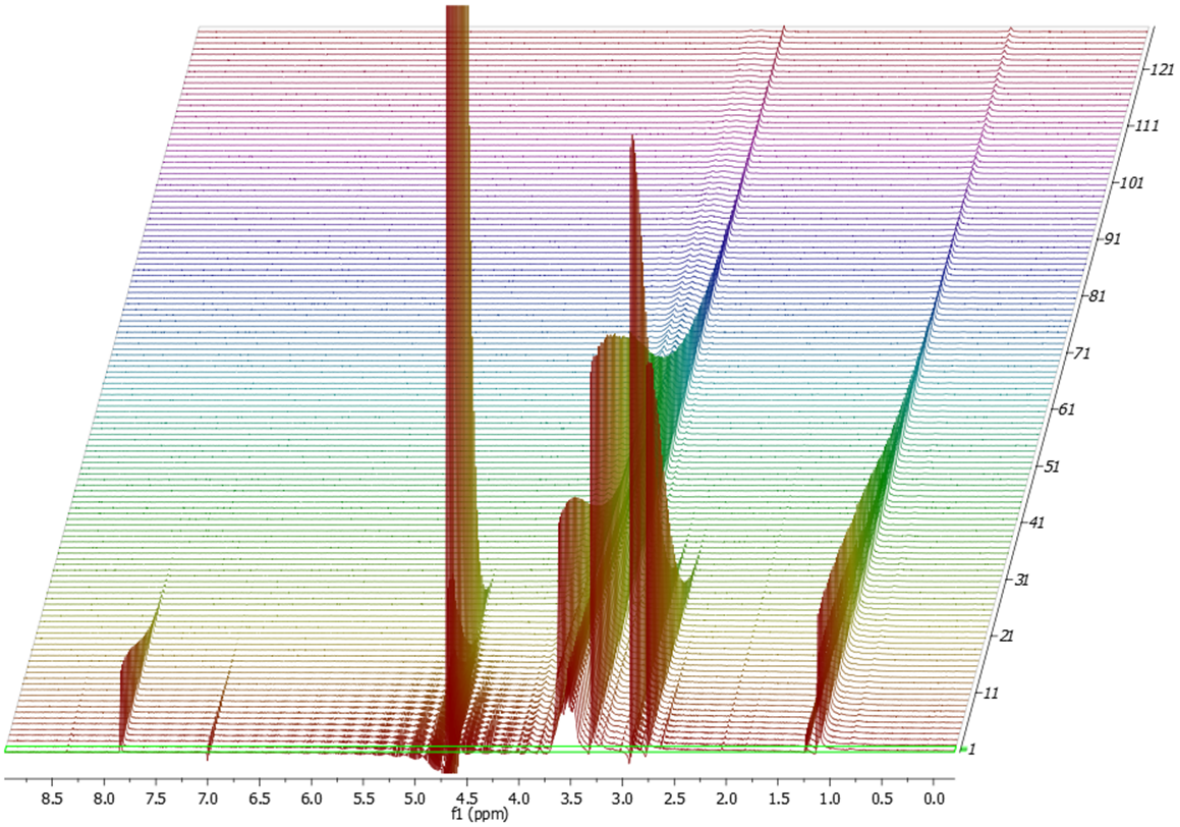

Fig. S1 1D-^{1}H-DOSY-NMR spectra of tNG measured in D_2O at 25 °C. Calculations were performed over 128 points of the corresponding signals.

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