Supporting Information

for Adv. Funct. Mater., DOI: 10.1002/adfm.201806693

Off-Stoichiometric Thiol-Ene Chemistry to Dendritic Nanogel Therapeutics

Yuning Zhang, Oliver C. J. Andrén, Randi Nordström, Yanmiao Fan, Martin Malmsten, Surinthra Mongkhontreerat, and Michael Malkoch*
Supporting Information

Off-Stoichiometric Thiol-Ene Chemistry to Dendritic Nanogel Therapeutics

Yuning Zhang,§ Oliver Carl-Janos Robert Per Oskar Andrén,§ Randi Nordström, Yanmiao Fan, Martin Malmsten, Surinthra Mongkhontreerat and Michael Malkoch*

Supplementary Methods

Materials

2,2-bis(hydroxymethyl) propionic acid (bis-MPA) was obtained from Perstorp. Di-methyl amino pyridine (99%) (DMAP), poly (ethylene glycol) Mw=20000 g mol\(^{-1}\) (PEG20k-OH) diethylether (analytical reagent grade) (Ether) were acquired from Fisher Chemicals. Dichloromethane (analytical grade) (DCM), tetrahydrofuran (THF) toluene-4-sulfonic acid (98%) (pTSA) were purchased from Merck. Pyridine (99.7%) was purchased from VWR. Mercapto propionic acid and 4-pentenoic acid was purchased from Sigma. PEG10k-G3-Allyl, PEG10k-hbG-G3-Allyl, PEG20k-G3-Allyl, mm-PEG5k-G3-Allyl, PEG5k-hbG3-Allyl, mm-PEG10K-G3-Allyl, mm-PEG10K-hbG3-Allyl was purchased and attained partly in kind as custom synthesis from Polymer Factory, Sweden.

Nuclear magnetic resonance (NMR)

Analysis were performed using a Bruker AM NMR. \(^1\)H NMR and \(^{13}\)C NMR were recorded at 400 MHz and 101 MHz respectively. \(^1\)H NMR spectra were acquired using a spectral window of 20 ppm, a relaxation delay of 1 second and 128 scans with automatic lock and shimming at a concentration of 10 mg mL\(^{-1}\). \(^{13}\)C NMR spectra were acquired using a spectral window of 240 ppm, a relaxation delay of 2 seconds and 2160 scans at a concentration of 100
mg mL$^{-1}$. Analyses of obtain spectra were conducted using MestReNova version 7.1.1-9649 (Mestrelab Research S.L 2012).

Size exclusion chromatography (SEC)

Analysis was performed in dimethylformamide (DMF) with LiBr (0.01 M) as the mobile phase at a flowrate of 0.2 mL min$^{-1}$ at 35 °C. ATOSOH EcoSEC HLC-8320GPC system was used equipped with an EcoSEC RI detector and three columns (PSS PFG 5μm; Microguard, 100 Å, and 300 Å) (MW resolving range: 300-100 000 Da) from PSS GmbH. Sample solutions with a concentration of 2.5 mg mL$^{-1}$ were used. A conventional calibration method was created using narrow linear poly (ethylene glycol) standards purchased from PSS range 106-44000 Da. Corrections for flow rate fluctuations were made using toluene as an internal standard. PSS WinGPC Unity software version 7.2 was used to process data and graphs where normalized and plotted in Origin 9.1.0 Sr1.

Dynamic Light Scattering (DLS)

The dynamic size of DNGs and DOX-DNGs were measured with a Malvern Zetasizer NanoZS at 37 °C in DI water. DNG samples were determined at polymer concentrations of 5 mg mL$^{-1}$ while DOX-DNGs were measured directly after purification. Each sample was allowed to equilibrate for 1 minute at 37 °C prior to analysis. All results are averages of minimum three individual samples where each sample data are an average of 3 measurements, each consisting of 10 runs. Data was processed using Malvern Zetasizer software v7.11.

Scanning electron microscopy (SEM)

Particles’ morphology and size was observed with SEM. Particles ( 500 μg mL$^{-1}$) were transferred on the silica plates and extensive dried before loaded to a Hitachi s4800 FE-SEM
and observed using 2kV acceleration voltage. The images were further analyzed with ImageJ software to measure the size of particles.

**Differential scanning calorimetry (DSC)**

Analysis was performed using a Mettler Toledo DSC820. A heating and cooling rate of 10 °C min\(^{-1}\) was used. Starting from room temperature (25 °C) the sample was heated to 120 °C, held there for 2 minutes, cooled to -30 °C, held there for 2 minutes and then heated to 120 °C. Analyses regarding midpoint Tg and midpoint Tm and ΔH were performed on the second heating cycle.

**General polycondensation procedure between bis-MPA and PEG.**

This was produced as published elsewhere.\(^{19}\) In short, PEG was added in a two necked round bottom flask equipped with argon inlet, magnetic stirrer and distillation equipment and heated to 130 °C. Every sixty minutes bis-MPA equivalent to one increase in dendritic generation was added along with pTSA (5 wt% pTSA based on bis-MPA added). During addition of bis-MPA, argon was flushed through the reaction vessel. When the desired generation had been reached one additional hour of argon flushing was applied after which vacuum was induced in the reaction vessel for 18 hours. The resin was extracted from the reaction vessel by dissolution in DCM and consecutively precipitated in ether two times and subsequent dried on high vacuum.

**Synthesis of PEG20K-hbG3-OH.**

Prepared according to general polycondensation procedure between bis-MPA and PEG, PEG20k-OH (5.00 mmol, 100 g), bis-MPA (14 eq, 70.0 mmol, 9.38 g), pTSA (5wt%, 469 mg). The product was collected as a white solid. (93.4%, 102.12 g) \(^1\)H NMR (400 MHz, MeOD, δ): 4.24-3.80 (q, 28H, -CH\(_2\)-OCO-, (bis-MPA)), 3.64 (q, 1828, CH\(_2\)-CH\(_2\)-O-, (PEG)),
1.18 – 1.14 (q, 42H, -CH₃, (Bis-MPA)). ¹³C NMR (100 MHz, DMSO-D₆, δ): 173.8 – 172.6 (1C, -COO-, (bis-MPA)), 69.71 (2C, CH₂-CH₂-O-, (PEG)), 63.6 (1C, -CH₂-, (bis-MPA)), 50.2 (1C, -COO-C-((CH₂-OH)₂, CH₃) (bis-MPA)), 49.5 (1C, -COOH-C-((CH₂-OR)₂, CH₃) (bis-MPA)), 48.1 (1C, -COO-C-(CH₂-OH, CH₃, CH₂-OR) (bis-MPA)), 46.2, 45.5 (1C, -COO-C-((CH₂-OR)₂, CH₃) (bis-MPA)), 16.7 (1C, -CH₃ (bis-MPA)). SEC (DMF) (Mw = 21688 g mol⁻¹, Mw=34671 g mol⁻¹, D=1.15). DSC (Tm=58.8°C).

Synthesis of PEG20K-hbG3-Allyl.

PEG20K-G3-OH (20g, 0.92 mmol) was dissolved in pyridine (3.60 mL, 44.26 mmol) and DCM (0.1 molar, 10 mL) in a round bottom flask equipped with magnetic stirrer. DMAP (360 mg, 2.95 mmol) was added and the reaction was cooled to 0 °C using an ice bath. 4-Pentenoic anhydride (5.39 mL, 29.5 mmol) dissolved in 5 mL of DCM was slowly added over a period of 30 minutes. Reaction was allowed to proceed overnight and progression was monitored by ¹³C NMR by confirming the presence of the Anhydride peak at 170.2 ppm. The crude reaction was precipitated in ether (1 L) and white powder collected, re dissolved in DCM and re precipitated in cold ether (1 L). Product was collected as a white powder (87%, 18.32 g) ¹H NMR (400 MHz, Chloroform-d, δ): 5.84 (ddt, J = 16.5, 10.2, 6.2 Hz, 16H), 5.18 – 4.91 (m, 34H), 4.40 – 4.31 (m, 8H), 4.29 – 4.20 (m, 32H), 3.66 (s, 6983H), 2.52 – 2.31 (m, 61H), 1.27 (s, 23H). SEC (DMF) (Mw = 22837 g mol⁻¹ Mw=29100 g mol⁻¹, D=1.12).

Synthesis Dendritic nanogels (DNG1-5).

PEG20K-hbG3-Allyl (200 mg, 8.75 µmol), Trimethylolpropane tris(3-mercaptopropionate) (TMP-SH) (amount according to desired cross-linking ratio, see Table S1) and 2,2-Dimethoxy-2-phenylacetophenone (DMPA) (20.6 mg) was dissolved in DCM (4 mL) in a round bottom flask. The solvent was slowly evaporated under reduced pressure to form a thin
transparent film. Milli-Q water (40 mL) was added to the flask and the flask was vortexed for 1 minute and submerged in an ultrasound bath (35 kHz) for 15 minutes. Resulting colloidal dispersion was exposed to UV light under constant stirring for 60 minutes (dosage = 1526 J mm\(^{-2}\)) (UVP Blak- Ray UV Benchtop Lamps, P/N: 95-00127-20M, 665nm, 230V, 50Hz. 3-Mercaptopropionic acid, 2-Mercaptoethylamine or 1-Hexanethiol (amount according to cross-linking ratio, see Table S1) along with DMPA (20.6 mg) dissolved in two mL of THF was added. The sample was vortexed for 1 minute and submerged in an ultrasound bath (35 kHz) for 15 minutes and subsequently exposed to UV light under constant stirring for 60 minutes (dosage = 1526 J mm\(^{-1}\)) (UVP Blak- Ray UV Benchtop Lamps, P/N: 95-00127-20M, 665nm, 230V, 50Hz). The liquid was filtered through a pour4 solid filter and poured in to a dialysis membrane and dialyzed against THF: H\(_2\)O (2:3), (1:3) and then water changing solvent with even intervals: 1, 1, 1, 2, 3, 15, 1 hours. Resulting slightly cloudy liquid was used as is or freeze dried to attain product as a fluffy cloud like solid (200 mg).

**Supplementary Results**

**Table S1.** Amounts used for cross-linking and functionalization during DNG synthesis and ratios

| Functionality | N\(_x\)-link | Cross-linker | n\(_{func}\) | Functionalization |
|---------------|--------------|--------------|--------------|------------------|
| COOH          | 10           | 11.6 mg, 29.1µmol | 6            | 27.7 mg, 262 µmol |
| NH\(_2\)      | 10           | 11.6 mg, 29.1µmol | 6            | 20.2 mg, 262 µmol |
| Hexyl         | 10           | 11.6 mg, 29.1µmol | 6            | 31.0 mg, 262 µmol |
| COOH          | 6            | 6.95 mg, 17.4 µmol | 10           | 46.3 mg, 460 µmol |
| NH\(_2\)      | 6            | 6.95 mg, 17.4 µmol | 10           | 33.6 mg, 460 µmol |
| Hexyl         | 6            | 6.95 mg, 17.4 µmol | 10           | 51.5 mg, 460 µmol |
| COOH          | 5            | 5.79 mg, 14.5 µmol | 11           | 50.9 mg, 480 µmol |
| NH\(_2\)      | 5            | 5.79 mg, 14.5 µmol | 11           | 37.0 mg, 480 µmol |
| Hexyl         | 5            | 5.79 mg, 14.5 µmol | 11           | 56.7 mg, 480 µmol |
| COOH          | 4            | 4.63 mg, 11.6 µmol | 12           | 55.3 mg, 523 µmol |
| NH\(_2\)      | 4            | 4.63 mg, 11.6 µmol | 12           | 40.4 mg, 523 µmol |
| Hexyl         | 4            | 4.63 mg, 11.6 µmol | 12           | 61.8 mg, 523 µmol |
| COOH          | 3            | 3.47 mg, 8.72 µmol | 13           | 60.1 mg, 567 µmol |
| NH\(_2\)      | 3            | 3.47 mg, 8.72 µmol | 13           | 43.7 mg, 567 µmol |
| Hexyl         | 3            | 3.47 mg, 8.72 µmol | 13           | 67.0 mg, 567 µmol |
Figure S1. **a)** Reproducibility of DNGs illustrated by five batches of DNG2-Allyl produced at five different occasions. **b)** Verification of DOX loading by fluorescence decrease.
Figure S2. Loading of gemcitabine and methotrexate as determined by $^1$H-NMR for DNG3-NH$_2$ and DNG3-COOH respectively. Gemcitabine spectra were collected in D2O and the loaded sample included 5.0 µM of benzyl alcohol as internal standard. Methotrexate spectra were collected in DMSO-D6 and the loaded sample included 3.3 µM of toluene as internal standard. Loading was calculated by integration followed by comparison to internal standard.

Figure S3. Cytotoxicity screening of pure DNGs on non-cancerous and cancer cell lines. AlamarBlue assay for 72 h.
Figure S4. Cytotoxicity of DOX loaded DNGs on non-cancerous cells, hDF and Raw 264.7. AlamarBlue assay for 72 h.
**Figure S5.** Co-localization of DOX and DOX-DNGs in 3D PANC1 cancer model. DOX signal was represented by red fluorescence; green fluorescence indicates either Calci-en-AM labeled living cells or lysoTracker DND-26 labeled lysosomes in cytoplasm; nuclei were stained with Hoechst 33342 and represented as blue signal. Scale bar = 100 µm.

**Table S2.** DLS and drug loading of DNGs

| Name         | Z-Average [nm] | PdI          | Drug yield [%] | Drug loading capacity (DLC) [m/m %] |
|--------------|----------------|--------------|----------------|------------------------------------|
| DNG3-Hexyl   | 196±6          | 0,114±0,01   | N/A            | N/A                                |
| DNG3-NH₂     | 193±3          | 0,122±0,021  | N/A            | N/A                                |
| DNG3-COOH    | 191±3          | 0,066±0,023  | N/A            | N/A                                |
| Compound          | Value  | Standard Deviation | Mean ± SD | Standard Error |
|-------------------|--------|--------------------|-----------|----------------|
| DOX-DNG3-Hexyl     | 194±18 | 0,306±0,015        | 23 ± 0.5  | 5.7            |
| DOX-DNG3-NH₂       | 183±12 | 0,341±0,019        | 21,9 ± 0.4| 5.5            |
| DOX-DNG3-COOH      | 231±16 | 0,071±0,02         | 22,4 ± 0.2| 5.6            |