Polymeric near-infrared absorbing dendritic nanogels for efficient in vivo photothermal cancer therapy

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Supplementary information

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1. Experimental details

1.1.Materials

The following chemicals were used as purchased: N-isopropylacrylamide (NIPAM), acryloyl chloride (AC, 96%, Fluka), extra dry dimethylformamide (DMF, 99.8%, Acros), triethylamine (TEA), ammonium persulphate (APS, 98%, Aldrich), sodium dodecyl sulphate (SDS, 98% Acros), N,N,N',N' – tetramethylenethylendiamine (TEMED, 99%, Aldrich), Aniline hydrochloride (Sigma, 97%)

1.2.Synthesis and physicochemical characterization

1.2.1. Synthesis of Acrylated Dendritic Polyglycerol (dPG-Ac 10%)

Dendritic polyglycerol with an average MW of 10 kDa was synthesized according to previously reported methodologies.[1] The dPG was dried overnight under vacuum at 140 ºC. A solution of acryloyl chloride (0.65 mmol, 52 μL) in dry DMF (1 mL) was added dropwise to a stirred solution of dPG (1 g, 10 kDa, 13.51 mmol OH equivalent) and TEA (1.08 mmol, 150 μL) in DMF (7 mL) at 0 ºC. Precipitation was observed. The reaction was left at room temperature for at least 4 h. A small amount of water was added to the reaction mixture in order to dissolve the precipitated salt. The reaction mixture was purified by dialysis (MWCO 2000) in water for at least 24 h. The above-mentioned acrylated dPG was preferably used directly after purification. Otherwise the product was stored in dark, at r.t in the presence of p-methoxyphenol and dialyzed again before usage. ^{1}HNMR (500 MHz, D_2O), δ: 3.2 – 4.3 (m, 5H, polyglycerol scaffold protons), 5.88 – 6.00 (m, 1H, vinyl), 6.08 – 6.28 (m, 1H, vinyl), 6.32 – 6.44 (m, 1H, vinyl).

1.2.2. Synthesis of NGs

The NGs were prepared following the procedure described by Cuggino et al.[2] In a regular synthesis, 100 mg of monomers, 67 wt% NIPAM, and 33 wt% of dPG-Ac, SDS (1.8 mg), and APS (2.8 mg) were dissolved in 5 mL of distilled water. Argon was bubbled into the reaction mixture for 15 min. The mixture was stirred under argon atmosphere for another 15 min. The reaction mixture was transferred into a hot bath at 68 ºC and polymerization was activated after 5 min with the addition of a catalytic amount of TEMED (120 μL). The mixture was stirred at 500 rpm for at least 4 h. The product was purified by dialysis membrane (MWCO 50 kDa) in water for 2 days and then lyophilized to obtain a white solid. ^{1}H NMR of 33 wt% dPG PNIPAM NGs: (500 MHz, D_2O), δ: 1.13 (s, 6H, isopropyl groups of NIPAM), 1.57 (2H, polymer backbone), 2.00 (1H, polymer backbone), 3.35 – 4.10 (6H, polyglycerol scaffold protons + 1H NIPAM).

1.2.3. Synthesis of sIPN NGs

The composites were synthesized following a two-step semi-interpenetration procedure. The dry NGs were immersed in an aniline HCl solution. After that, an equimolar amount of ammonium persulphate (APS) (90 mg, 0.3 mmol) was added to the NG as an oxidant to produce the conducting polymer inside the gel. This way the linear NIR absorbing polymer formed and interpenetrated the gel. Different concentrations of aniline and APS as well as polymerization times were tried (Table 1). The effective semi-interpenetration was tested by UV-Vis spectroscopy, ^{1}HNMR, and FT-IR spectroscopy. The product was dialyzed for 2 days (MWCO 50 kDa) in water and stored in solution. ^{1}H NMR: (500 MHz, D_2O), δ: 1.13 (s, 6H, isopropyl groups of NIPAM), 1.57 (2H, polymer backbone), 2.00 (1H, polymer backbone), 3.35 – 4.10 (6H,

polyglycerol scaffold protons + 1H NIPAM), 7.20-7.50 (4H, aromatic protons of PANI).

The size of the nanoparticles was studied by Dynamic Light Scattering (DLS) using a Nano-ZS 90 Malvern equipped with a He–Ne laser (λ =633 nm) under scattering of 173º. The shape of the NGs was studied by Cryogenic Transmission Electron Microscopy (cryoTEM) and Atomic Force Microscopy (AFM). The cryoTEM measurements were carried out on a Philips CM12 TEM (FEI company, Oregon, USA) using the Gatan (Gatan Inc., California, USA) cryoholder and stage. 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. Data were analyzed using NanoScope Analysis 1.3 software. NG aqueous dispersions (2 mg mL^{-1}) were spin coated on a Mica sheet at 90 rps for 5 min. Samples were analyzed by Nano World tips, Point Probe® Plus Non-Contact / Tapping Mode - Long Cantilever - Reflex Coating (PPP-NCLR20), with resonance frequency of 190 kHz and force constant of 48 N m^{-1}. The phase transition temperature was determined by UV-Vis spectroscopy on a Cary 100 Bio UV-Vis spectrophotometer equipped with a temperature-controlled, six-position sample holder and DLS.

1.2.4. Photothermal effect

The photothermal effect of the nanocomposites was studied as a function of time and concentration. Different concentrations of PNIPAM-dPG/PANI were prepared (20, 40, 60, and 80 mg mL^{-1}), 200 µL of each solution was placed into a well in a 96-well plate and irradiated with a fluence of 2W cm^{-2}. An infrared diode laser module at 785 nm (FC-D-785 CNI) was used and the temperature was recorded at different time points with an infrared camera (FLIR E30, 25° optic, 60 Hz).

1.3.Biological studies

1.3.1. Cell culture

A2780 cells were routinely maintained in RPMI 1640 with 2 mM glutamine (Life Technologies), 10% FCS (Biochrom AG) and 1% penicillin/streptomycin (Life Technologies) at 37 °C and 5% CO_2.

1.3.2. MTT Assay

1 x 10^5 cells mL^{-1} were seeded into 96-well plates and incubated overnight at 37 °C and 5% CO_2. The next day, medium was replaced for 50 µL fresh medium and 50 µl dilutions of the test compounds (in triplicates). After 48 h of incubation, the cells were washed three times with 200 µL PBS per well and 10 µL MTT (5 mg mL^{-1} stock solution in PBS) was added in 100 µL fresh medium. After incubation for 4 h at 37 °C and 5% CO_2, the supernatant was discarded and 100 µL isopropanol 0.04 M HCl was added. Absorbance was read in a Tecan Infinite M200 Pro microplate reader at 570 nm. Assays were repeated three times independently. Relative cell viabilities were calculated by dividing the average absorbance values by the absorbance value of untreated cells.

1.3.3. Photothermal therapy *in vitro*

1 x 10^5 cells mL^{-1} were seeded into a 75 cm^{-2} culture flask and 0.1 mg mL^{-1} PNIPAM-dPG/PANI (6.7 mg mL^{-1} stock solution in PBS) was added. Control cells were seeded and treated similarly but incubated with medium only.

During incubation, a 50 µL sample was taken from the cell culture medium every day and stored at 4 °C until measurement. The concentration of PNIPAM-dPG/PANI in cell culture medium was determined by absorbance measurements at 595 nm to indirectly monitor cell uptake.

After 64 h of incubation, cells were washed twice with 10 mL PBS and trypsinized. Detached cells were centrifuged for 5 min at 138 x g, resuspended in medium, and counted. Aliquots of 1 x 10^6 cells were centrifuged again for 2 min at 300 x g, resuspended in 100 µL medium, transferred to 0.2 mL PCR-tubes, and centrifuged again for 2 min at 300 x g. The supernatant was aspirated and the cell pellet was irradiated with a 785 nm laser from above the tube with the lid open. Temperature of the pellet was monitored from the side of the tube using an infrared camera, and the laser power was adjusted so that the temperature was kept constant around 42 +/- 1 °C (240-260 mW). Once 42 °C was reached, which usually took about 1.5 - 2 min, the stop watch was started and irradiation continued for 5 min. Parallel samples were incubated in a 42 °C water bath for the same time. In a second set of samples, cell pellets were irradiated or heated for 5 min as before, but not once but four times with 3 min breaks in between. Control cells which had not been incubated with PNIPAM-dPG/PANI were irradiated/heated the same way but since they did not reach 42 °C, they were just irradiated with the same current and time as the PNIPAM-dPG/PANI incubated cells.

After irradiation/heating, the cell pellets were resuspended in 1.5 mL medium, diluted 1:10 into 96-well plates (20 µL cell suspension in 200 µL total), and incubated for 24 h at 37 °C and 5% CO_2. Relative cell viability was determined using MTT assay.

1.4.In vivo studies

The studies were performed in accordance with all national or local guidelines and regulations as described in the approved Tierversuchsantrag A 0452/08 (Landesamt Berlin für Gesundheit) from 28.11.2012 for the EPO GmbH Berlin-Buch.

1.4.1. Tolerability

The tolerability of the PNIPAM-dPG/PANI NGs was studied in female nude (NMRI, nu/nu) mice in two ways. First, the mice were treated with a single intravenous (i.v.) injection of the test sample at different doses of 10, 20, 40, 70, and 100 mg kg^{-1}. In the second part, the mice were treated with the maximum tolerated dose found in part 1 once daily for 5 days. In both cases, the body weight was measured 3 times per week during three weeks to evaluate the toxicity of the NGs.

1.4.2. Therapeutic effect

For the photothermal therapy *in vivo* female nude (NMRI, nu/nu) mice with an ovarian xenograft A2780 tumor were used. A2708 tumor transplants were obtained from tumors, cut into small pieces (~2x2x2 mm) and transplanted subcutaneously into female nude mice (Taconic) on day 0. The mean tumor volume at this time was 0.086 - 0.088 cm^3. Two different protocols were followed for studying the therapeutic effect *in vivo*. In the first approach, female nude (NMRI, nu/nu) mice were treated with a single dose of 10 mg kg^{-1} that was intratumorally injected and irradiated with a 785 nm laser for 5 min with a fluence of 2W cm^{-2}. The temperature was monitored with an infrared camera. In the second approach, female nude (NMRI, nu/nu) mice were treated with a single dose of 100 mg kg^{-1} that was intravenously injected and irradiated after 6, 24, or 48 h in the same conditions as before. Several controls were used in both approaches: (i) PBS, (ii) PNIPAM-dPG, (iii) PNIPAM-dPG + NIR, and (iv) PNIPAM-dPG/PANI.

Three times a week body weight and tumor volume were measured. The experiment was completed on day 39.

2. Supplementary figures

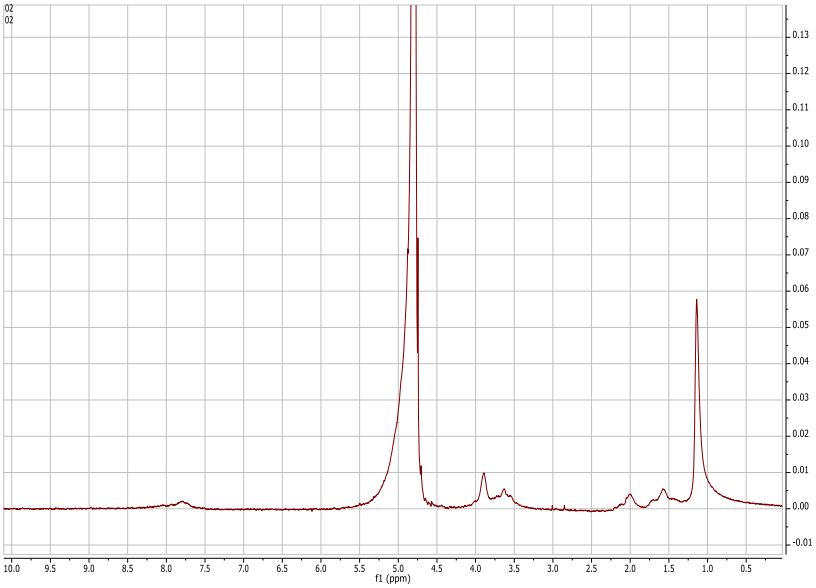

Figure S1. ^{1}H NMR of PNIPAM-dPG/PANI.

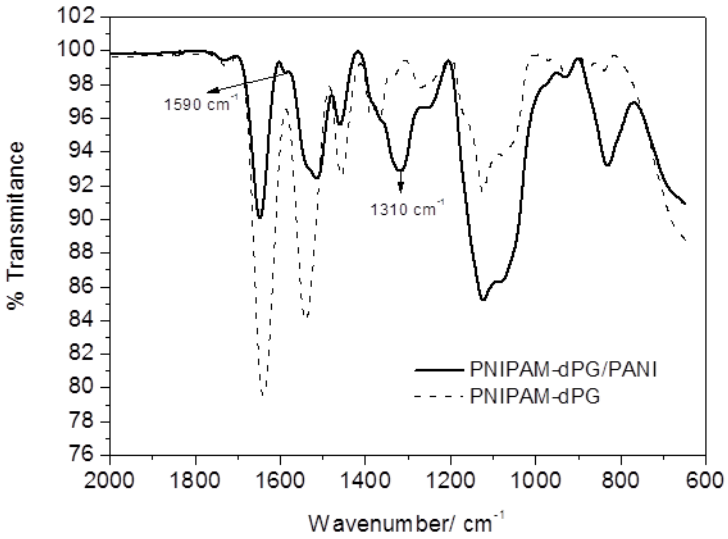

Figure S2. FTIR of PNIPAM-dPG (dashed line) and PNIPAM-dPG/PANI (line).

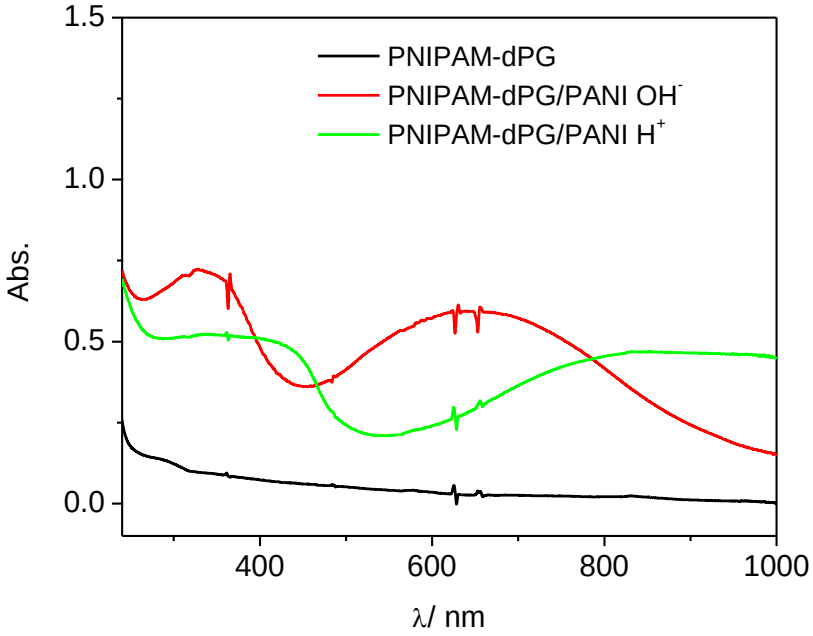

Figure S3. UV spectra of PNIPAM-dPG (black line), PNIPAM-dPG/PANI in acidic (green line), and basic pH (red line).

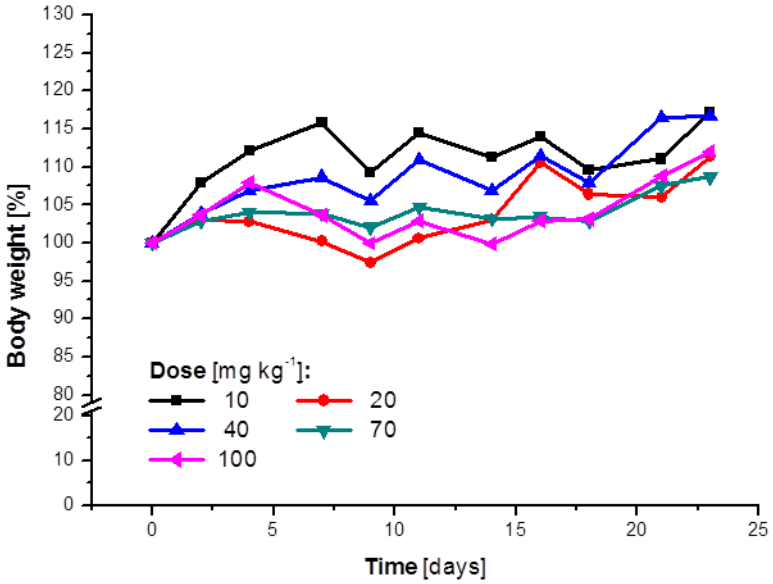

Figure S4. Tolerability *in vivo*. Body weight change after a single treatment with different doses of PNIPAM-dPG/PANI.

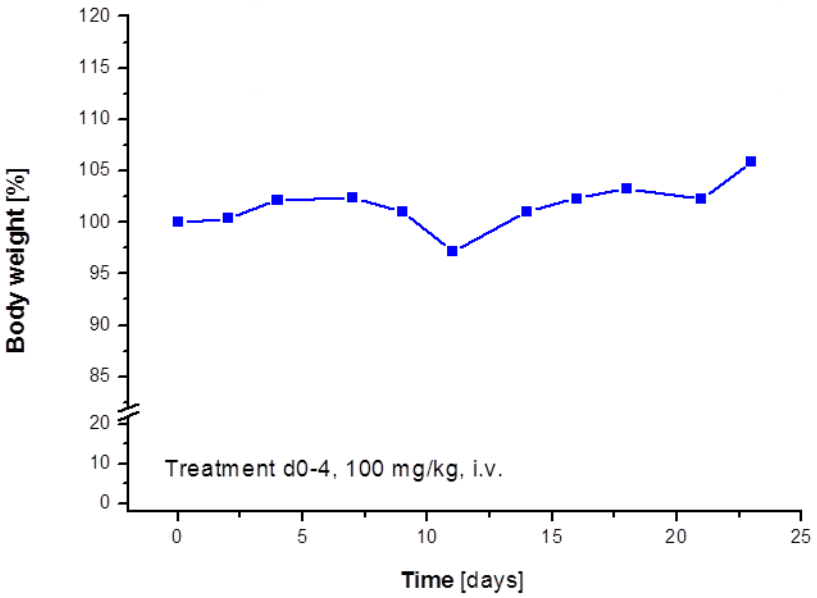

Figure S5. Tolerability *in vivo*. Body weight change after repeated treatments of d0-d4 with 100 mg kg^{-1} of PNIPAM-dPG/PANI.

3. References

[1] R. Haag, H. Tuerk, S. Mecking, Google Patents, **2003**.
[2] J. C. Cuggino, C. I. Alvarez I, M. C. Strumia, P. Welker, K. Licha, D. Steinhilber, R.-C. Mutihac, M. Calderon, *Soft Matter* **2011**, *7*, 11259-11266.