Electronic Supplementary Material (ESI) for Polymer Chemistry.

Supplementary data

Engineering Thermoresponsive Polyether-Based Nanogels for Temperature Dependent Skin Penetration

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Materials

The following chemicals were used as purchased: acryloyl chloride (AC, 96% Fluka), triethylamine (TEA, 99% Acros), dry dimethylformamide (DMF, 99.8% Acros), poly(ethylene glycol) methyl ether methacrylate (OEGMA475, molecular weight 475 g mol^{-1}, Aldrich), di(ethylene glycol) methyl ether methacrylate (DEGMA, molecular weight 188 g mol^{-1}, Aldrich), ammonium persulphate (APS, 98% Aldrich), sodium dodecyl sulphate (SDS, 98% Across), tetramethylenethylendiamine (TMEDA, 99% Aldrich), rhodamine B hydrochloride (95% Aldrich), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDCI, 98% Aldrich), 4-(dimethylamino)pyridine (DMAP, 99% Aldrich).

Methods

Synthesis of NGs

1. Synthesis of acrylated dendritic polyglycerol

Dendritic polyglycerol (dPG) with average molecular weight of 9 kDa (PDI = 1.3) was synthesized according to previously reported methodologies.[1] For the synthesis of the acrylated derivative (dPG-Ac), acryloyl chloride (98 mg, 0.087 mL, 1.08 mmol) was added dropwise to a stirred cold solution of dPG (1 g, 9 kDa, 13.5 mmol OH equivalent) and TEA (328 mg, 0.450 mL, 3.24 mmol) in DMF (6 mL) at 0 °C. The reaction was allowed to reach room temperature at 25 °C and run for 4 h. The reaction mixture was then diluted with water and purified by dialysis in water for 2 days using benzoylated dialysis tubings (2 kDa molecular weight cut-off MWCO, Sigma-Aldrich). The obtained colorless dPG-Ac solution was stored with a stabilizer (p-methoxy phenol) at 4 °C. An aliquot (1 mL) of the purified

product solution was taken out and dried on a rotation evaporator at 30 °C. The dried aliquot was further used for characterization via NMR and for determining the reaction yield and conversion ratio (attached functional groups). Conversion ratio: > 95 %. Yield: > 95%. ^{1}H NMR (400 MHz, D_2O, δ): 3.00 – 4.00 (5 H, m, dPG scaffold protons), 6.05 (1 H, s, vinyl, H^a), 6.25 (1H, s, vinyl, H^b), 6.46 (1H, s, vinyl, H^c). The spectrum is disclosed in Figure S6.

2. Synthesis of tNGs

In a typical procedure for the synthesis of tNG, variable amounts of DEGMA, OEGMA475, dPG-Ac (monomers and crosslinker in total 190 mM), SDS (1.32 mM), and APS (2.54 mM) were dissolved in distilled water in a 50 mL flask so that the overall reaction volume did not exceed 5 mL at room temperature. The mixture was bubbled with argon for 10 min and then stirred for 15 minutes under argon atmosphere at 600 rpm, following a heating on an IKA-RCT basic heater at maximum heating rate to 78 °C. Just after reaching 70 °C the polymerization process was initiated by adding a catalytic amount of a TMEDA solution (320 mM). The mixture was stirred at 600 rpm at the adjusted temperature under argon atmosphere for 4 h. After reaction, the product was dialyzed for 2 days in water using regenerated cellulose dialysis tubes (50 kDa MWCO, Spectra/Por) and then lyophilized to obtain the colorless rubber-like product. The yield of tNG synthesis varied between 76 and 88 %. ^{1}H NMR (400 MHz, D_2O, δ): 0.92 - 1.47 (3 H, m, -CH_3 of OEG methacrylic backbone, H^b), 1.60 – 2.20 (2 H, m, -CH_2- of OEG methacrylic backbone, H^a), 3.42 (3 H, s, -O-CH_3 of OEG chain, H^h) 3.45 - 4.10 (5H, m, tPG protons), 3.60 – 3.80 (24 H, m, -O-CH_2-CH_2-O- of OEG chain, H^d + H^e + H^f + H^g), 4.15 – 4.25 (2 H, m, -COO-CH_2-CH_2-O- of OEG chain, H^c) (Figure S7). FT-IR: ν (cm^{-1}) = 3368, 2874, 2346, 1726, 1645, 1453, 1352, 1248, 1100, 861 (Figure S8). Physico-chemical properties and characteristics are mentioned in Table S1.

3. Synthesis of RhdB labeled tNGs

Labeling of tNGs was accomplished by the coupling of RhdB to the dPG precursor by carbodiimide-mediated ester formation. Therefore, a solution of EDCI (132.6 mg, 0.7 mmol, 5 eq.), DMAP (84.5 mg, 0.7 mmol, 5 eq.) in 5 mL DMF were mixed with a rhodamine B (265 mg, 0.55 mmol, 4 eq.) solution in 4 mL DMF and stirred for 10 min at room temperature. The rhodamine B mixture was added to a cold dPG (1 g, 0.14 mmol OH equivalent, 1 eq.) solution in 3 mL DMF and stirred for 5h. The reaction mixture was diluted with water and dialyzed for 2 days in water using benzoylated dialysis tubings (2 kDa MWCO, Sigma-Aldrich). Rhodamine B-dPG (Rhd-dPG) conjugate formation was confirmed by chromatography on thin layer chromatography (n-butanol/methanol/water, v/v/v 9:3:1), and appearance of a faster band on a Sephadex G 25 fine column. The polymer-dye conjugate was lyophilized to yield **NG20** (54%). Conjugation efficiency by UV-Vis (Figure S9): 32%. Dye per dPG ratio: 1.3. Rhd-dPG was acrylated in the already described methodology to yield Rhd-dPG-Ac.

For the synthesis of thermoresponsive rhodamine B labeled tNG (**NG20**), DEGMA, Rhd-dPG-Ac, and dPG-Ac (monomers and crosslinker in total 190 mM) were polymerized via radical precipitation polymerization as described above. For the synthesis of RhdB labeled NG, not exhibiting thermoresponive behavior below 80°C, (**NG21**), DEGMA, OEGMA475, and dPG-Rhod-Ac (monomers and crosslinker in total 190 mM) were polymerized via the same method but using 90°C heat bath temperature. Since this NG did not exhibit a volume to phase transition temperature (VPTT) in DLS in the range of 17-75°C, these nanogels have been labeled as non-thermoresponsive (**NG21**).

Chemical structure characterization

^{1}H NMR analysis was performed using a Bruker 400 MHz NMR spectrometer. The sample preparation, in which 8 mg of sample had been dissolved in 0.8 mL of D_2O, was performed 24 h prior to the measurement. FT-IR analysis was carried out using a JASCO FT-IR 4100 LE spectrophotometer in the range of 4000–500 cm^{-1}.

Purification methods

Benzoylated regenerated cellulose membranes: MWCO = 2.000 Da (Sigma-Aldrich), and MWCO = 50.000 Da (SpectraPor) were 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) was performed with Sephadex G 25 Fine and Sephacryl S-100 HR from GE Healthcare. The material was activated by swelling in the respective eluent prior to performing chromatography.

Dynamic light scattering measurements

Size, size distribution, and thermoresponsive behavior of tNGs were measured at various temperatures ranging from 15 to 80 °C by DLS 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.

Transmission electron microscopy (TEM)

Transmission electron microscopy samples were prepared on copper grids (200 meshes) by blotting samples in 1% aqueous uranyl acetate and visualized using a Philips CM12 Electron Microscope. 1 mg of each nanogel was dissolved in 1 mL of deionized water one day before the TEM experiment. The excess of water was evaporated in an oven at 30 °C for 2 min.

Atomic force microscopy (AFM)

The AFM tapping mode images were recorded with a MultiMode 8 AFM equipped with a Nanoscope V controller from Veeco Instruments, Santa Barbara, California. The results were analyzed by means of Nano-Scope Analysis 1.3 software. Nanogel solution (1 mg * mL^{-1}) was dropped on a HOPG sheet (Plano, Product#G3389C, 0,8°+-0,2°, 10x10x1mm) and incubated for 10 min. Supernatant was sucked away until a thin fluidic film was remaining on top of the HOPG. Finally, glass probe holder for scanning in fluid (Bruker, Product# MTFML) on a clamped Cantilever C (Bruker, Pruduct#SNL-10, C-f_0: 40-75 kHz; k: 0.24 N/m) was linked to the nanogel bearing HOPG sheet.

VPTT determination by DLS measurements

On the Nano-ZS 90 Malvern instrument, volume to phase transition temperature (VPTT) was defined as the temperature at the inflection point of the Z-average curves.

Cloud point temperature (Tcp) determination by UV-Vis transmitance

Cloud points were measured on a Cary 100 Bio UV-Vis spectrophotometer equipped with a temperature control. For Tcp measurements buffer phosphate pH 7.4 nanogels solutions (1 mg mL^{-1}) were run through 3 heating and cooling cycles at 0.5 °C min^{-1} while monitoring both the transmittance at 450 nm (1 cm path length) and the solution temperature (from 18 to 60 °C). The temperature of the sample was determined by the internal temperature probe. The cloud point (Tcp) of each nanogel was defined as the temperature at the inflection point of the normalized transmittance curves.

Cytotoxicity assays

Cytotoxicity of the nanogels was evaluated through impedance measurement by the xCELLigence real-time cell analyzer (RTCA) from Roche Applied Science (Mannheim, Germany) and the MTS assay CellTiter 96 $AQ_{ueou}s$ One Solution Cell Proliferatin Assay (Promega, Madison, USA) according to the manufactures' protocols. HaCaT cells (human skin keratinocyte cell line) cultured in Roswell Park Memorial Institute medium 1640 (RPMI 1640) supplemented with 10% fetal bovine serum, 600 mg ml^{-1} L-glutamine, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin and NIH3T3 cells (human skin fibroblast cell line) cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum,300 mg ml^{-1} L-glutamine, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin were seeded in a 96-well E-plate (10.000 cells per well) and incubated at 37 °C, 5% CO_2, and 99% humidity. The plate was placed in the RTCA device and the impedance was measured at least every 15 minutes. After 20 h the plate was removed and the nanogel was added at defined concentrations ranging from 5 mg mL^{-1} to 100 ng mL^{-1}. Non-treated and doxorubicin

(100μM) treated cells served as a control. The plate was placed back and impedance measurements continued for another 48 h. Growth curves were generated by the RTCA software 1.2.1. Following the real time measurement, 20 μL MTS solution (5 mg mL^{-1} in PBS) was added to each well. After 3 h incubation the medium containing the MTS solution was transferred in a new 96-well plate, and the optical density was measured in a microplate reader at a test wavelength of 490 nm and a reference wavelength of 630 nm. IC50 values were calculated with the GraphPad Prism 5.01 software using end point data obtained from impedance measurements or the MTS assay, respectively.[2]

Cellular uptake

Cellular uptake of rhodamine B labeled nanogels was monitored by confocal laser scanning microscopy (cLSM). For the uptake study 50.000 A549 cells (adenocarcinomic human alveolar basal epithelial 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 6h and 18 h, respectively. Cells were grown at 37 °C, 5% CO_2, and 99% humidity and maintained in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum (Biochrom AG, Berlin, Germany), 100 U mL^{-1} penicillin, and 100 μg mL^{-1} 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% Triton X-100 for 5 min and washed 2 times with PBS. To detect co-localization with late endosomes and lysosomes, samples were incubated for 1 h in the dark at 37 °C with anti-hLAMP1 antibody (10 μg mL^{-1}) and for another 1 h our with FITC-labelled anti-mouse IgG2b (10 μg mL^{-1}). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (2,5 μg mL^{-1}) and F-actin

(cytoskeleton) with Atto 647N labeled phalloidin (0,133 µM). Cells were observed and imaged using a confocal laser scan microscope (Leica DMI6000 CSB stand).

Tissue samples

Human skin samples were obtained from healthy volunteers undergoing plastic surgery, within 24 hours after surgical excision. The study was approved by the Institutional Ethics Committee of the Medical Faculty of the Charite-Universitätsmedizin Berlin (approval EA1/135/06), and written informed consent was obtained in accordance with GCP-ICH guidelines and Declaration of Helsinki Principles.

Topical application of nanogel preparation on skin explants

Skin samples were examined macroscopically to detect tissue damage. Subcutaneous fat was removed and skin was stretched and tightened by means of needles on rigid sheets. Temperature responsive (**NG20**) and non-temperature responsive (**NG21**) nanogels (41.3 mM final concentration rhodamine B) were applied on 1 cm², leaving safety margins of 0.5 cm to the border of the tissue in order to avoid non-specific sideways penetration. **NG21** and **NG20** samples (n = 4 (n represents the number of samples per group, i. e. 4 skin pieces for each type of nanogel, and for the control), 1 donor) were incubated at 4° or 37°C for 4 hours in a humidified chamber, respectively. After incubation, remaining material on the skin surface was removed by means of adhesive tapes (5x).

Cryosections and fluorescence microscopy

The penetration profile of nanogels was investigated on cryosections by fluorescence microscopy. The treated skin area was split into four blocks, which were frozen in liquid nitrogen. Cryosections of 5 μm thickness were prepared from each block using a microtome (2800 Frigocut-N, Reichert-Jung, Heidelberg, Germany). Samples were then observed under fluorescence microscopy (Olympus BX60F3). Pictures of at least 20 randomly chosen skin sections per skin sample were taken and analyzed. In approximately 25% of skin sections fluorescence intensity was detected also in the epidermis. Skin sections were divided into two groups according to the skin area where fluorescence intensity was observed, i.e. skin sections with fluorescence localized only in the stratum corneum only and skin sections with fluorescence detectable in stratum corneum and also in the epidermis. Three different skin areas were analyzed: stratum corneum, epidermis, and dermis. The mean fluorescence intensity (MFI) of each area was calculated using the ImageJ software.

MFI statistics

For statistical analysis the Excel software (Microsoft, USA) was used. Differences between the MFI of groups were analyzed by a two-tailed unpaired T-test; $p \leq 0.05$ was regarded as indicating a significant difference. Moreover one-way ANOVA was used to compare the data of the different groups. There was a statistical significant difference between the four groups ($F = 6,49$, $df = 3$, $p = 0.015$) in the epidermis , whereas no statistical differences were found in the stratum corneum ($F = 0.66$, $df = 3$, $p = 0.600$) and dermis ($F = 2,58$, $df = 3$, $p = 0.126$).

Table S1

Physico-chemical properties and reaction ratios for tNGs.

Reference	Sample	SDS (mg)	dPG-Ac(/RhdB-dPG-Ac) feed [wt%]	dPG-Ac [%]	χ_{DEGMA}	DLS Intensity size diamter [nm] at 15°C[a]	DLS PDI value[a]	VPTT [°C][a] determined by DLS
Figure 1b	**NG01**	1	10	12	1	170 ± 11	0.20 ± 0.08	33 ± 3
	NG02	2	10	12	1	146 ± 24	0.19 ± 0.09	38 ± 5
	NG03	4	10	12	1	141 ± 15	0.23 ± 0.05	30 ± 4
	NG04	8	10	12	1	118 ± 7	0.25 ± 0.04	32 ± 3
	NG05	16	10	12	1	112 ± 13	0.21 ± 0.05	30 ± 4
Figure 1c	**NG06**	2	5	7	0.923	272 ± 2	0.07 ± 0.01	42 ± 3
	NG07	2	10	7	0.923	140 ± 2	0.10 ± 0.01	42 ± 2
	NG08	2	20	7	0.923	111 ± 1	0.14 ± 0.02	44 ± 2
	NG09	2	45	7	0.923	97 ± 4	0.26 ± 0.01	46 ± 3
	NG10	2	55	7	0.923	106 ± 4	0.30 ± 0.04	52 ± 3
Figure 1d	**NG11**	2	5	7	1	220 ± 4	0.07 ± 0.02	23 ± 1
	NG12	2	5	7	0.962	183 ± 1	0.09 ± 0.02	32 ± 2
	NG13	2	5	7	0.923	272 ± 20	0.20 ± 0.01	42 ± 3
	NG14	2	5	7	0.716	143 ± 19	0.50 ± 0.05	50 ± 4
Figure 1e	**NG15**	2	10	4	1	165[b] ± 10	0.29 ± 0.03	42 ± 3
	NG16	2	10	6	1	155[b] ± 12	0.19 ± 0.07	39 ± 4
	NG17	2	10	8	1	152[b] ± 9	0.23 ± 0.08	39 ± 4
	NG18	2	10	10	1	149[b] ± 14	0.16 ± 0.07	37 ± 6
	NG19	2	10	12	1	146[b] ± 24	0.19 ± 0.09	38 ± 5
	NG20	2	8.4/1.6	9	1	80 ± 2	0.18 ± 0.01	36 ± 1
	NG21	2	0/10	9	0.910	78 ± 1	0.12 ± 0.01	-

[a] Average value of 3 measurements of one synthesized tNG batch; [b] Average value of 3 measurements of two equally synthesized tNG batches.

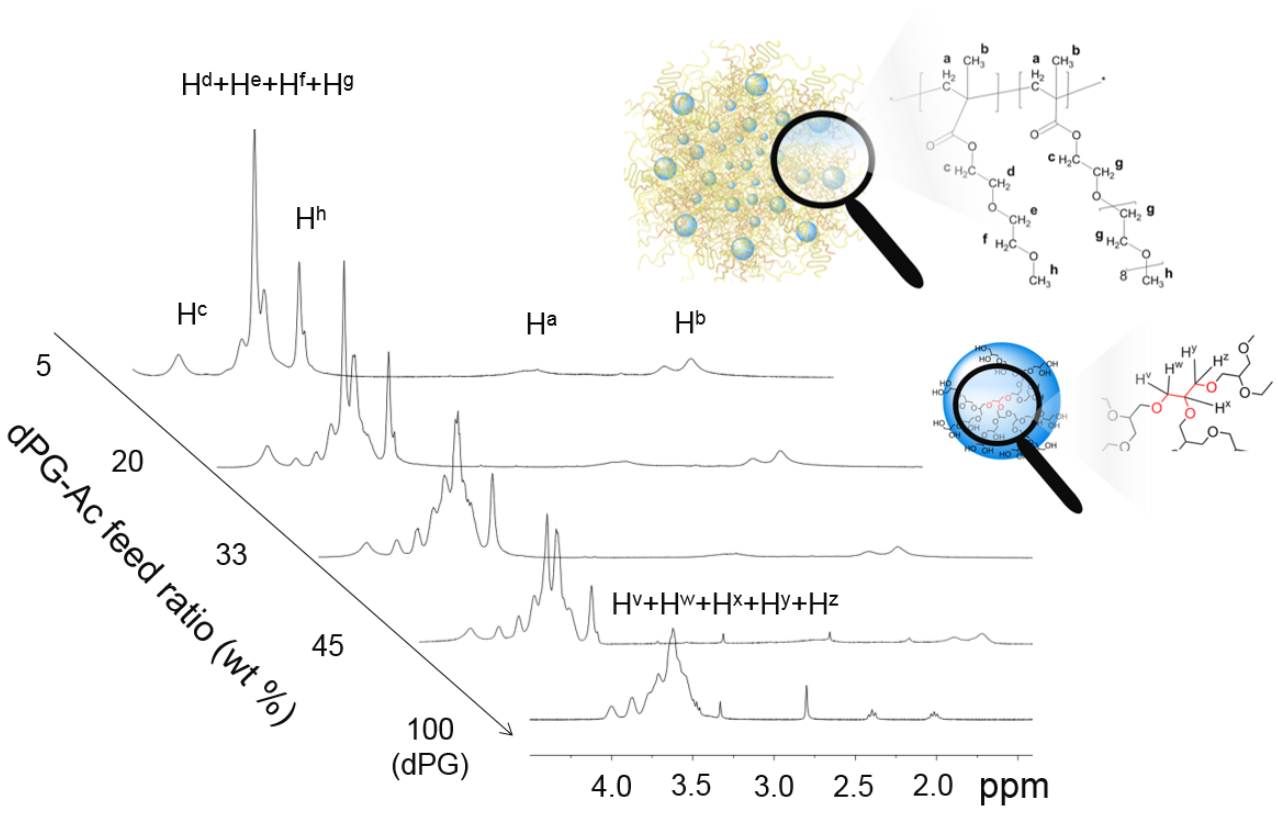


Fig. S1. ^{1}H NMR spectra (D_2O, 400 MHz) of tNGs with increasing dPG-Ac composition. Decrease of OEG signals ($H^d+H^e+H^f+H^g$) signals at 3.7 ppm correspond to ethylene glycol chain protons and increase of dPG backbone signal ($H^v+H^w+H^x+H^y+H^z$) at 3.5 - 4.0 ppm indicate an increase in tNG's crosslinker composition.

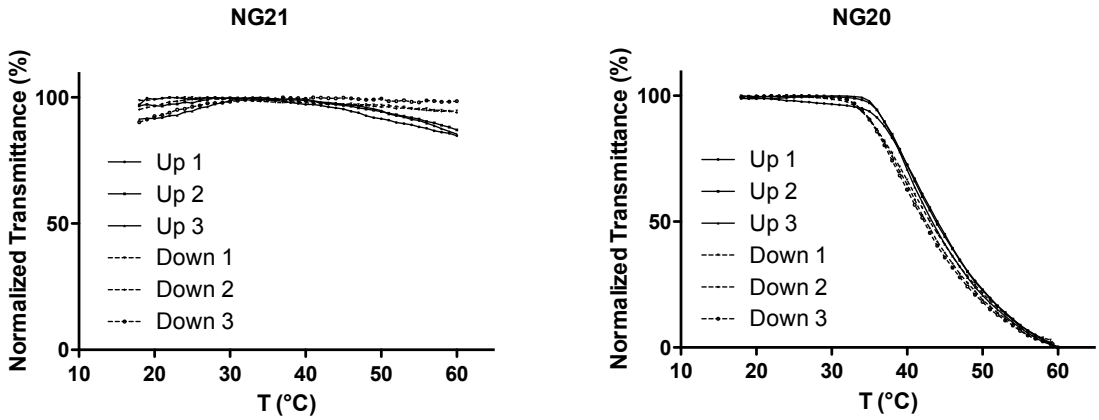
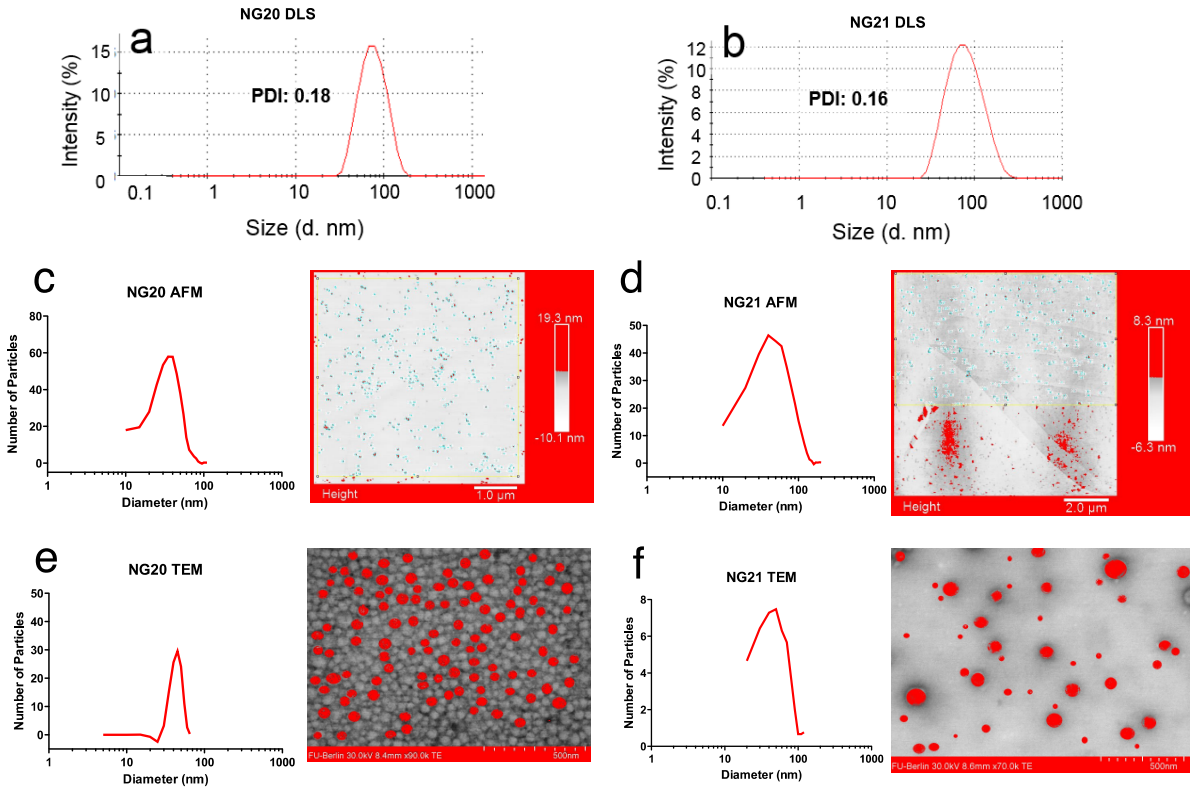


Figure S2. Cloud point temperature determination by UV-Vis measurements of NG20 and NG21, in three cooling-heating cycles.



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Sample	Technique	Mean Diameter (nm)	PDI
NG20	DLS	80 ± 2	0.18
	AFM	38 ± 15	
	TEM	45 ± 7	
NG21	DLS	78 ± 1	0.12
	AFM	58 ± 29	
	TEM	53 ± 23	

Fig. S3. Size distribution by dynamic light scattering measurements (a, b), AFM (c, d) and TEM (e, f) of **NG20** (a, c, d) and **NG21** (b, d, f). (g) Statistical mean diameter comparison between AFM, TEM, and DLS measurements.

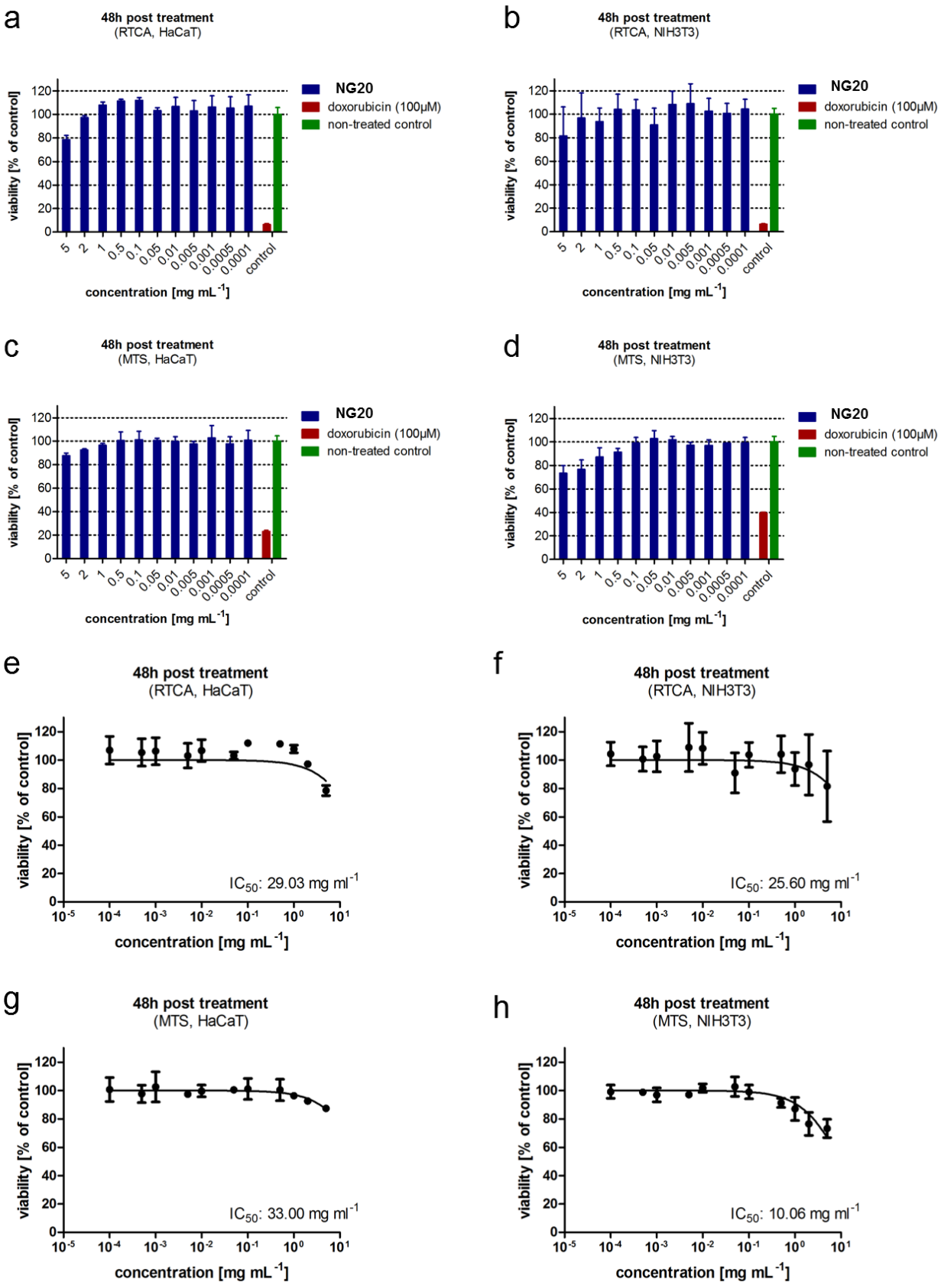

Fig. S4: Cytotoxicity assays of NG20 by cell proliferation (RTCA; a, b, e, f) and metabolic activity (MTS assay; c, d, g, h) and on human keratinocyte cell line (HaCaT; a, c, e, g) and fibroblast cell line (NIH3T3; b, d, f, h) were analyzed 48 h post treatment.

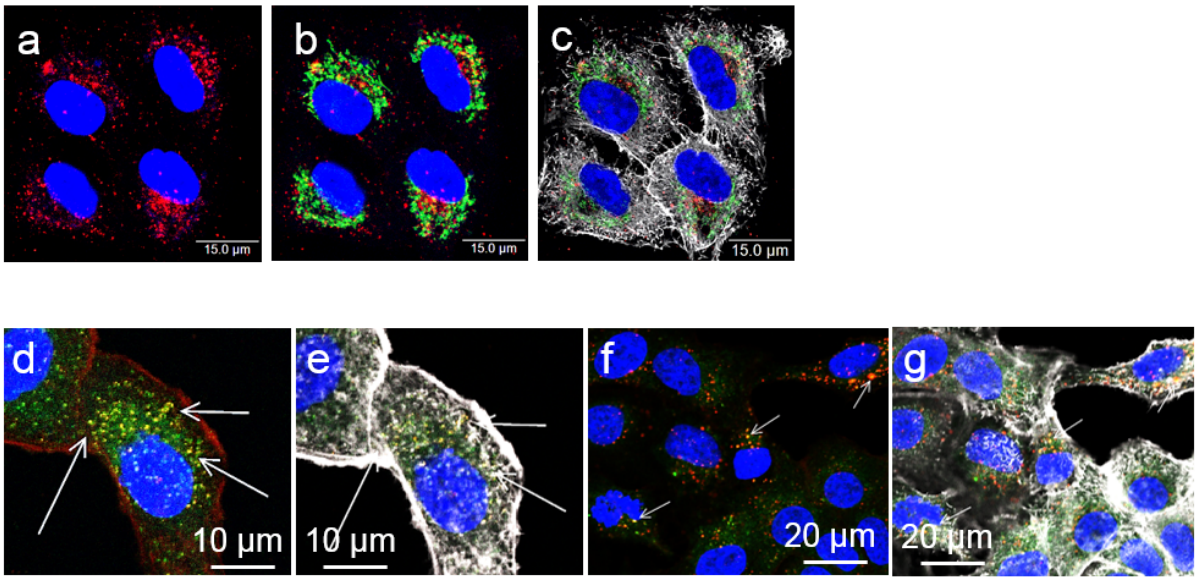

Fig. S5: Cell uptake of tNGs into A549 cells. (a) Uptake was observed for rhodamine B labeled nanogels **NG21** after 18h incubation (a-c). (b) Green areas represent early endosomes stained with EEA1 FITC labeled antibody. (c) Blue areas represent the DAPI stained nucleus of the cells while grey areas (c, e, g) mark the Atto 647N phalloidin stained cytoskeleton (F-actin) as shown in the merged image. Co-localization was observed when late endosomes were stained with LAMP1/CD107a FITC labeled antibody (green areas, d-g) after 6h (d,e) and 18h (f,g) incubation.

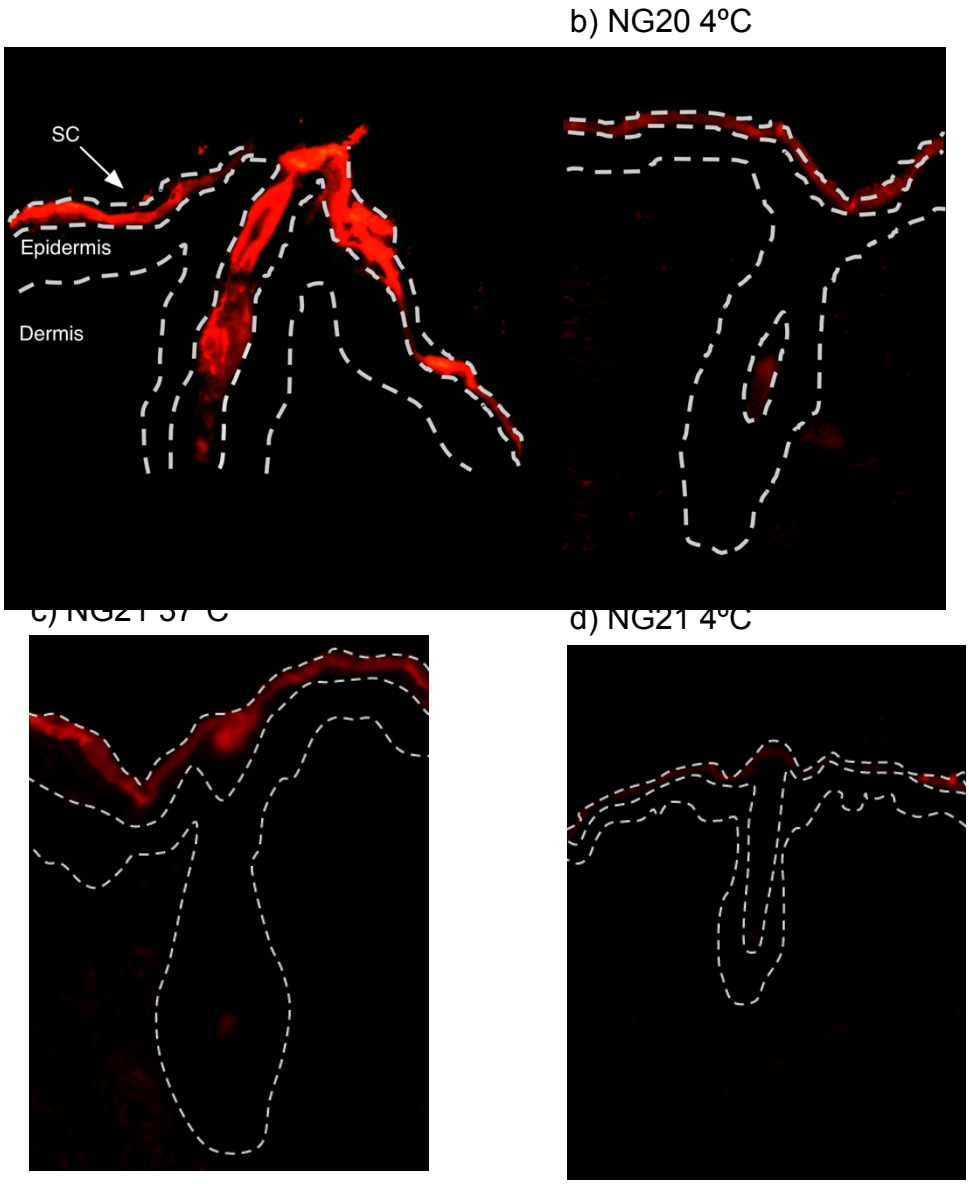

Fig. S6: Internalization of **NG20** (a, b) and **NG21** (c,d) into HF channels at 37 (a, c) and 4 ºC (b, d).

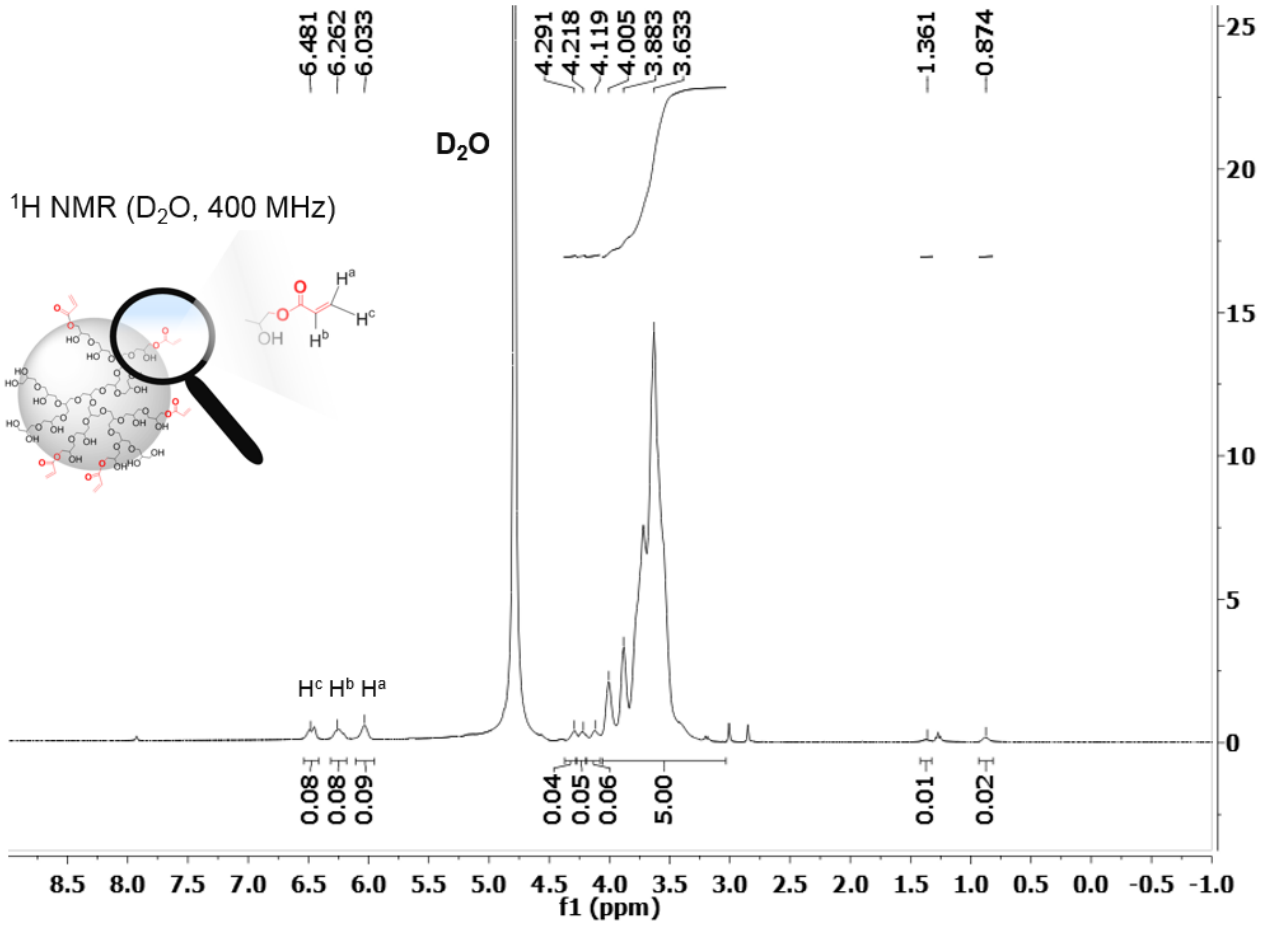

Fig. S7. ^{1}H NMR spectrum (D_2O, 400 MHz) of dPG-Ac

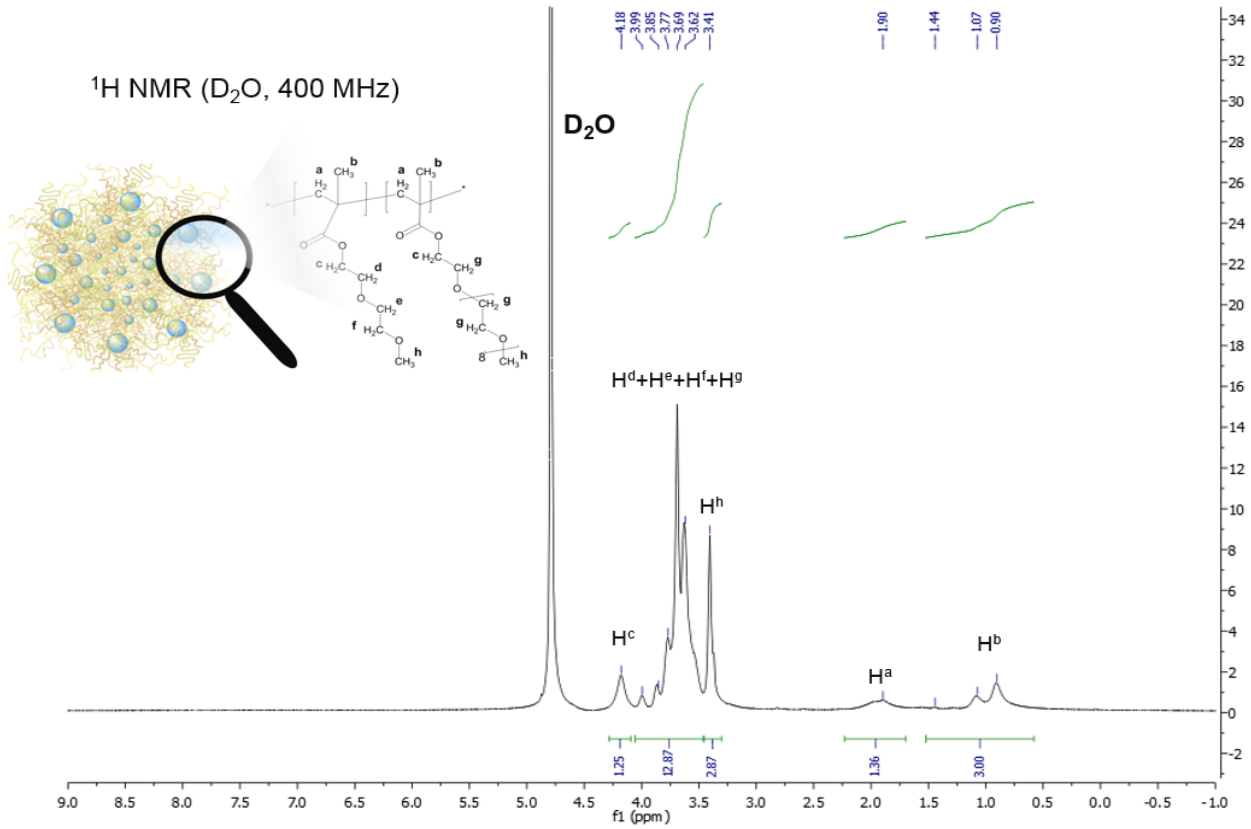

Fig. S8. ^{1}H NMR spectrum (D_2O, 400 MHz) of tNG.

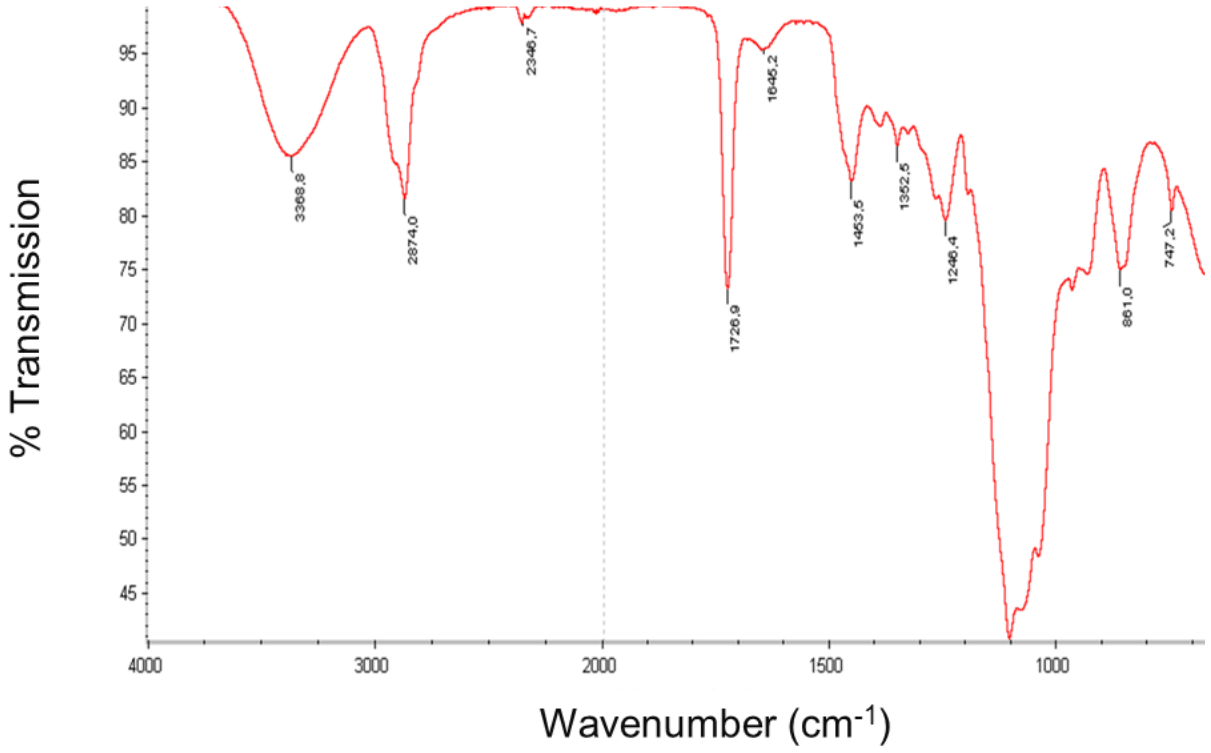

Fig. S9. FT-IR spectrum of tNG.

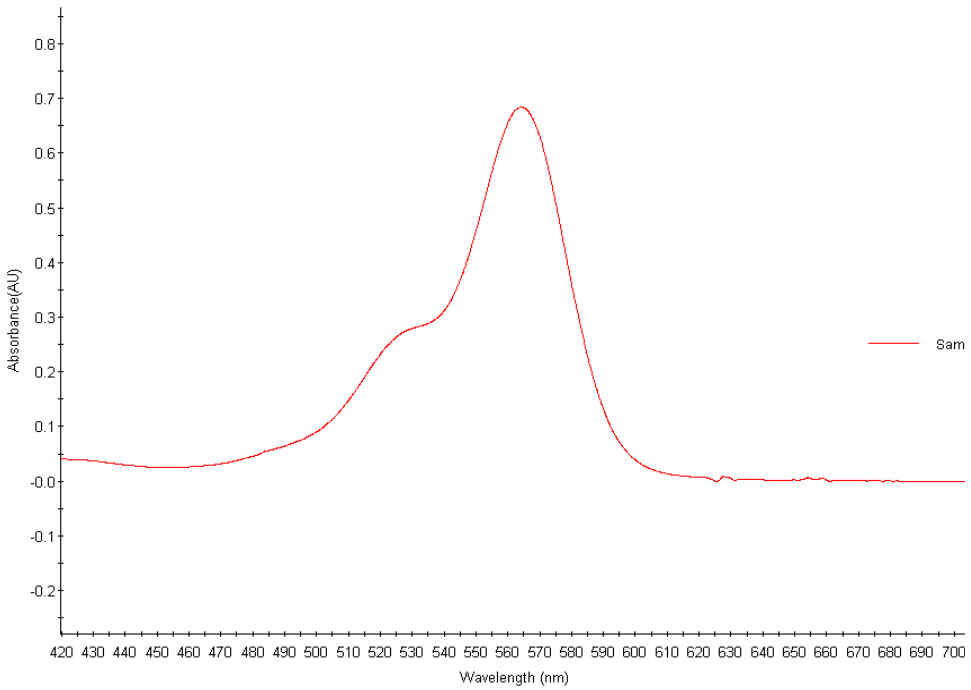

Figure S10. UV-Vis spectrum of rhodamine B labeled tNG

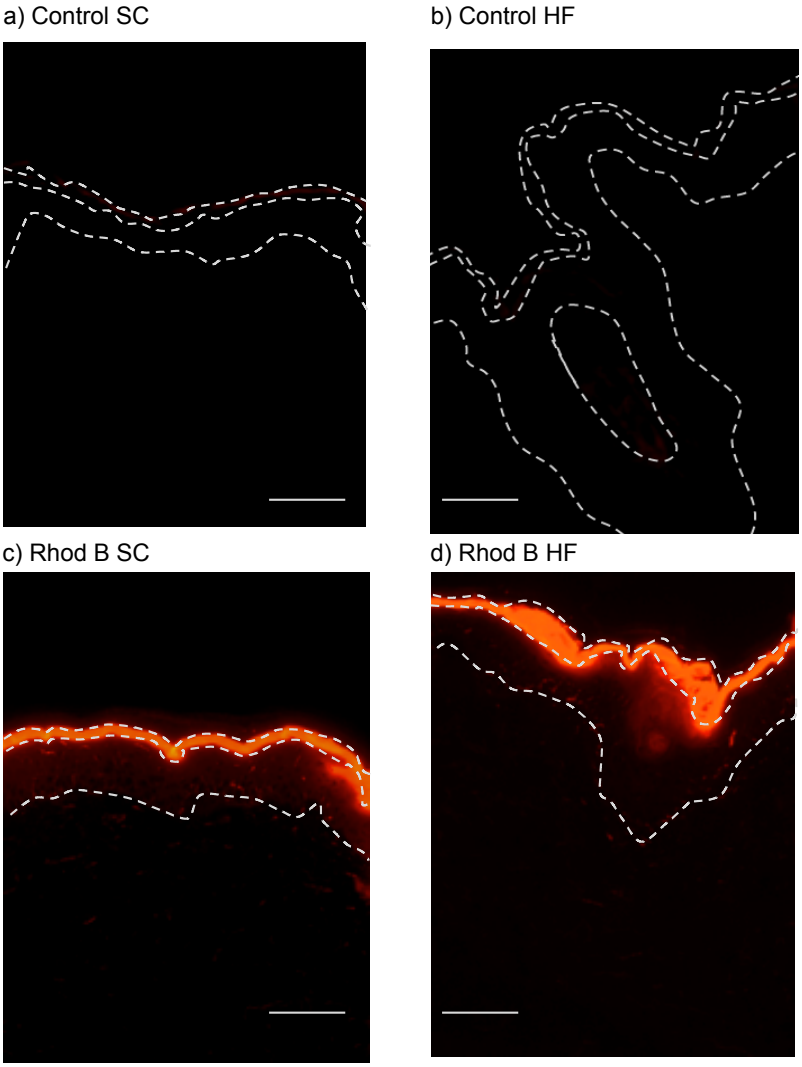

Figure S11. Non fluorescence (a, b) and Rhod B (c, d) controls in SC (a, c) and HF (b, d). All bars denote 100 nm.

References

[1] Haag R, M. S., Tuerk H. Deutsche Patenanmeldung DE 10211664 A1. 2004.

[2] Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55-63.