

Supporting Information for

# One-pot synthesis of doxorubicin-loaded multiresponsive nanogels based on hyperbranched polyglycerol

Ana Sousa-Herves,<sup>a</sup> Stefanie Wedepohl,<sup>a</sup> and Marcelo Calderón<sup>a\*</sup>

<sup>a</sup> *Institut für Chemie und Biochemie, Freie Universität Berlin, Takustrasse 3, 14195 Berlin, Germany.*

## Table of Contents

Materials and methods	S3
Dynamic Light Scattering	S3
Atomic Force Microscopy	S3
Nanoparticle Tracking Analysis	S4
Synthesis of hyperbranched polyglycerol (hPG) acrylate	S4
Synthesis of 2-(5,5-dimethyl-1,3-dioxan-2-yloxy) ethyl acrylate (DMDEA)	S4
General procedure for the preparation of unloaded NGs	S5
Table S1	S5
Figure S1	S6
Figure S2	S6
Figure S3	S7
Stimuli-responsive behavior of the NGs	S7
General procedure for the preparation of DOX-loaded NGs	S7
Figure S4	S8
HPLC characterization of DOX treated under free radical polymerization conditions	S8

Figure S5	S9
In vitro DOX release studies	S9
Figure S6	S9
MTT assays	S10
Figure S7	S11
Table S2	S11
Real time cell analysis	S11
Figure S8	S12
Cellular internalization studies	S14
Figure S9	S15
References	S16

**Materials and methods.** Solvents were purchased from Sigma Aldrich and used as received unless otherwise stated. Column chromatography was performed with alumina for column chromatography. Thin-layer chromatography (TLC) was done on alumina plates. Acryloyl chloride, OEGA, BMADS, and sodium dodecyl sulfate (SDS) were purchased from Sigma Aldrich. Doxorubicin hydrochloride (DOX·HCl) was purchased from Yick-Vic Chemicals & Pharmaceuticals (China). AIBN was obtained from Fluka and recrystallized from methanol. Dialysis tubes with a molecular weight cutoff (MWCO) of 100 Da and 50 kDa were obtained from Spectrum. Amicon Ultra-15 centrifugal filters (MWCO 50 KDa) were purchased from Millipore. Sephadex G25 was obtained from GE Healthcare. For ESI measurements, a TSQ 7000 (Finnigan Mat) instrument was used. FT-IR analysis was carried out using a JASCO FT-IR 4100 LE spectrophotometer. NMR experiments were performed in a Bruker DRX 500 instrument. NMR chemical shifts are reported in ppm ( $\delta$  units) downfield from the  $\text{CDCl}_3$  signal.  $\text{CDCl}_3$  was filtered through  $\text{K}_2\text{CO}_3$  or basic alumina prior to NMR experiments. NMR spectra were analysed with MestReNova software.

**Dynamic Light Scattering.** DLS measurements were carried out on a Malvern Nano ZS (Malvern Instruments, U.K.) operating at 633 nm or at 532 nm with a  $173^\circ$  scattering angle. NG solutions were filtered (0.45  $\mu\text{m}$ , nylon) before any measurement being performed.

**Atomic Force Microscopy.** NGs were filtered through a centrifugal filter device (Amicon Ultra-15, 50 K) and washed 3 times with water to remove the salts before performing AFM measurements. Samples were spin coated on a Mica sheet at 90 rps for 5 min. The AFM imaging was performed in air using a MultiMode 8 AFM equipped with a NanoScope V system (Veeco, Santa Barbara, CA) operated in tapping mode. Samples were analysed by Nano World tips, Non-Contact/Tapping Mode-Long Cantilever (NCL-W), with resonance frequency of 190 kHz and force constant of  $48 \text{ N m}^{-1}$ . Statistical analyses were performed using NanoScope Analysis 1.3 software in a  $1.7 \times 1.7 \mu\text{m}$  image (for NG 3). Total Count: 92. Diameter: 45.3 nm. SD: 17.459 nm. In the case of hydrolyzed NG 3 (Fig. 2), statistical analysis was done in a  $3.8 \times 3.8 \mu\text{m}$  image. Total Count: 45. Diameter: 166.06 nm. SD: 46.362 nm.

**Nanoparticle Tracking Analysis.** Size and concentration of NGs were measured by NTA using a Nanosight NS500. The samples were prepared by diluting the solutions prepared for DLS measurements 1000 times with water and immediately measured at 25 and 55 °C. Particle sizes and concentration are given as the average of 3 measurements.

**Synthesis of hyperbranched polyglycerol (hPG) acrylate.** hPG-acrylate (35% acrylation) was obtained by acrylation of hPG following a procedure described by Haag and coworkers.<sup>1</sup> Briefly, hPG (1.05 g,  $M_n$  = 10 kDa, 14.05 mmol of OH groups, 1 eq) was dissolved in 12 mL DMF and triethylamine (1.9 mL, 14.0 mmol, 1 eq) was added. The solution was cooled down to 0°C and a solution of acryloyl chloride (0.77 g, 8.54 mmol, 0.6 eq) was slowly added in 3 mL DMF. The solution was stirred for 12 h at rt. The precipitate was filtered off and the solution was dialyzed (MWCO 2 kDa) with  $\text{CHCl}_3$  for three days. The product (896 mg, 85 % yield) was stored at 4 °C in  $\text{CHCl}_3$ . <sup>1</sup>H NMR (500 MHz,  $\text{CDCl}_3$ ): 6.41-6.44 (m, 0.35 H,  $\text{CH}_2=\text{CH}-$ ), 6.12-6.17 (m, 0.35 H,  $\text{CH}_2=\text{CH}$ ), 5.84-5.86 (m, 0.35 H,  $\text{CH}_2=\text{CH}-$ ), 3.51-4.19 (m, 5 H, hPG). The acrylation degree was determined from the <sup>1</sup>H NMR. hPG with acrylation degrees up to 50 % could also be prepared.

**Synthesis of 2-(5,5-dimethyl-1,3-dioxan-2-yloxy) ethyl acrylate (DMDEA).** DMDEA was synthesized following a procedure similar to one described in the literature.<sup>2</sup> Briefly, 2,2-dimethylpropan-1,3-diol (6.25 g, 0.06 mol), trimethyl orthoformate (6.47 g, 0.06 mol) and pyridinium-*p*-toluenesulfonate (TsOH-py, 0.15 g, 0.6 mmol) were dissolved in 15 mL dry  $\text{CH}_2\text{Cl}_2$  and stirred overnight at rt. Then, the solution was extracted with an aqueous 10% NaOH solution (3 times), dried with  $\text{K}_2\text{CO}_3$ , and concentrated under vacuum. The intermediate (8.60 g, 98% yield) was characterized by <sup>1</sup>H NMR, which was consistent with previously reported results.<sup>2</sup> Subsequently, the intermediate (4.0 g, 27.36 mmol) was dissolved in dry toluene (30 mL). 2-hydroxyethyl acrylate (4.13 g, 35.6 mmol), 4-methoxyphenol (66.4 mg, 1.6 wt %) and TsOH-py (138.2 mg, 0.55 mmol) were added and the solution was stirred at 80°C for 12 h. After evaporating the solvent, the residue was purified by column chromatography with basic alumina (hexane/ ethyl acetate 5:1). After solvent evaporation, DMDEA was obtained as a colorless oil (4.93 g, 40% yield). <sup>1</sup>H NMR in  $\text{CDCl}_3$  (filtered through basic alumina) was

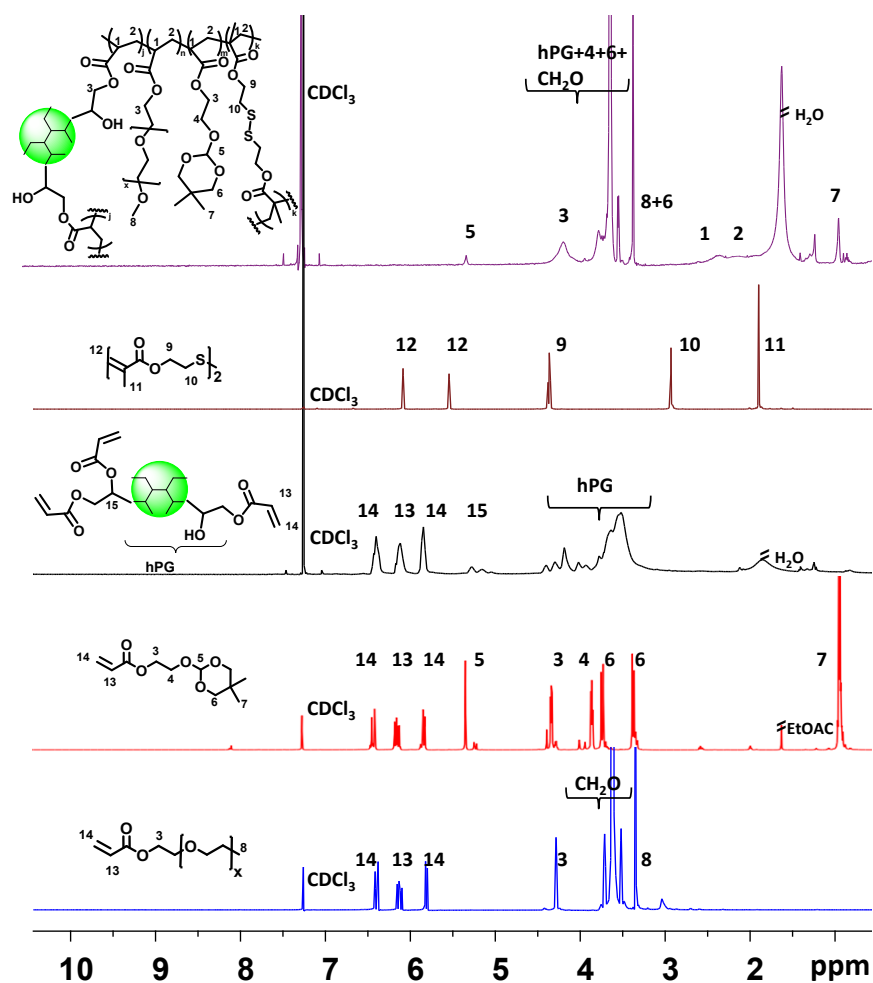
in agreement with reported data and confirmed the purity of DMDEA. <sup>1</sup>H-NMR (500 MHz, CDCl<sub>3</sub>): 6.41-6.45 (m, 1H, CH<sub>2</sub>=CH-), 6.13-6.18 (m, 1H, CH<sub>2</sub>=CH-), 5.83-5.85 (m, 1H, CH<sub>2</sub>=CH-), 5.35 (s, 1H, CHO<sub>3</sub>-), 4.34-4.36 (m, 2H, -CO<sub>2</sub>-CH<sub>2</sub>CH<sub>2</sub>O-), 3.87-3.89 (m, 2H, -CO<sub>2</sub>-CH<sub>2</sub>CH<sub>2</sub>O-), 3.74-3.77 (d, 2H, -OCH<sub>2</sub>CMe<sub>2</sub>CH<sub>2</sub>O-), 3.38-3.40 (d, 2H, -OCH<sub>2</sub>CMe<sub>2</sub>CH<sub>2</sub>O-), 0.97 (s, 6H, -C(CH<sub>3</sub>)<sub>2</sub>).

**General procedure for the preparation of unloaded NGs.** Unloaded NGs were synthesized by free radical polymerization using a nanoprecipitation technique to template NGs formation. OEGA and BMADS were filtered through basic alumina before being employed. NGs with different monomer ratios, and control NGs lacking one of the monomers were prepared (Table S1). As a representative example, the synthesis of NG 3 was as follows: hPG acrylate (12.5 mg, ~ 1 μmol), AIBN (3 mg, 18.2 μmol), DMDEA (16 mg, 70 μmol), OEGA (14.17 mg, 29.52 μmol), and BMADS (0.57 mg, 1.96 μmol) were dissolved in 0.5 mL DMF. The organic mixture containing the monomers, crosslinkers and initiator was subsequently precipitated into 10 mL of a 50 mM phosphate buffer pH 8.0 solution which contained SDS (5 μM). The mixture was bubbled with Ar for 45 min and subsequently heated to 65 °C overnight. After that time, the reaction flask was opened to air to quench the reaction, and the mixture was dialyzed (MWCO 50 kDa) in PB buffer pH 8 (to ensure stability of the orthoester groups) for 3 days, yielding NGs with mass recoveries around 30 %. The NGs were kept in solution at 4 °C or freeze-dried before further characterization.

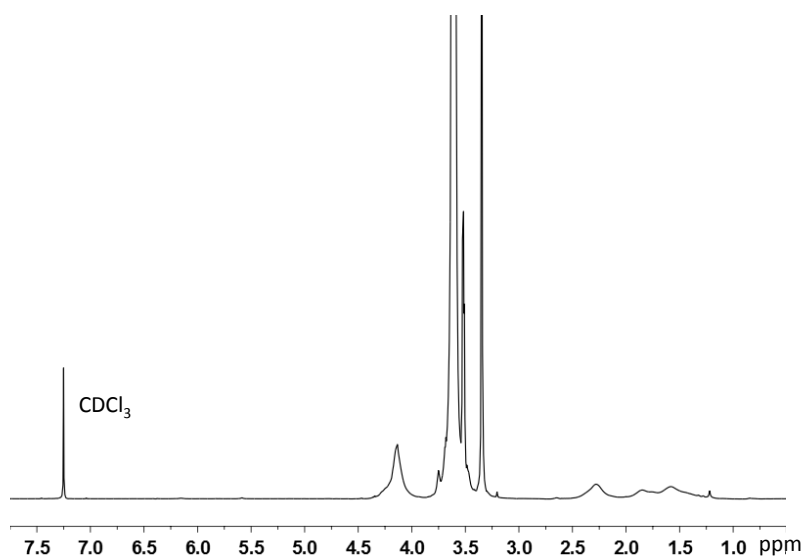
**Table S1**

Entry	DMDEA/OEGA/BMADS/hPG <sup>a</sup>	hPG (wt%)	Concentration Monomer/Crosslinkers <sup>b</sup>	Size <sup>c</sup> (nm)	PDI <sup>c</sup>
NG 1	70/30/2/0.5	12.5%	0.2 M	90	0.2
NG 2	70/30/2/1	25%	0.2 M	97	0.15
NG 3	70/30/2/0.5	12.5%	0.4 M	87	0.11
NG 4	140/30/2/0.5	12.5%	0.34 M	176	0.13
NG 5	0/30/2/1	50%	0.06 M	115	0.27
NG 6	70/0/2/1	50%	0.14 M	115	0.24
NG 7	70/30/0/1	25%	0.2 M	160	0.21
NG 8	70/30/2/0	0%	0.2 M	N.d. <sup>d</sup>	N.d. <sup>d</sup>

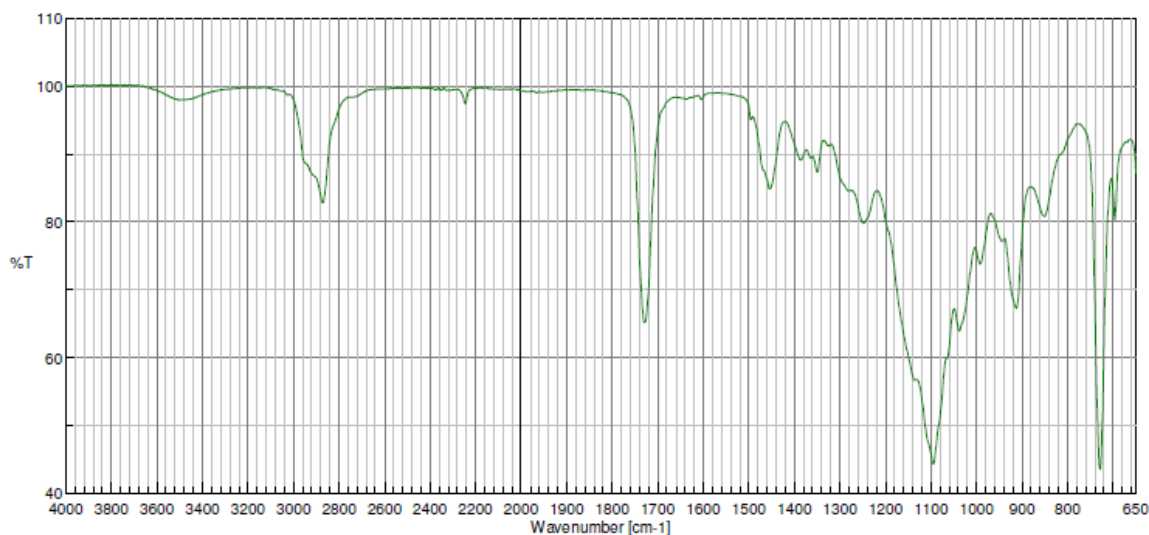
a) Molar ratio. b) Concentration in the organic phase. c) Determined by DLS. d) Not determined, NGs could not be obtained.



**Figure S1.**  $^1\text{H}$  NMR spectra (CDCl<sub>3</sub>) of NG 3 and individual monomers and crosslinkers.



**Figure S2.**  $^1\text{H}$  NMR spectrum (CDCl<sub>3</sub>) of NG 5, lacking DMDEA monomer.



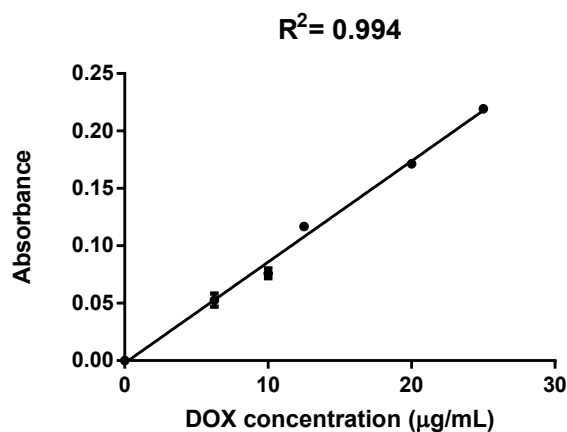
**Figure S3.** FT-IR spectrum of NG 3, showing the bands of C-O vibration at  $1740\text{ cm}^{-1}$  and the lack of the characteristic vinyl bands at  $1640\text{ cm}^{-1}$ .

**Stimuli-responsive behavior of the NGs.** The pH-sensitive behavior of the NGs was analysed by DLS, AFM, and  $^1\text{H}$  NMR.  $50\ \mu\text{L}$  of a solution of acetate buffer  $5\ \text{M}$  pH 5 were added to  $950\ \mu\text{L}$  of a filtered solution of the NGs (with and without DMDEA) in PB  $10\ \text{mM}$  pH 8. The resulting solutions were immediately measured by DLS (without any further filtering). For AFM measurements, the salts were previously removed by centrifugal filters. To investigate the sensitivity towards DTT,  $50\ \mu\text{L}$  of a solution of DTT ( $15\ \text{mg/mL}$ ) were added to  $950\ \mu\text{L}$  of a filtered solution of the NGs (with and without BMADS) and measured by DLS.

**General procedure for the preparation of DOX-loaded NGs.** DOX·HCl was dissolved at  $7.5\ \text{mg/mL}$  in MeOH.  $\text{Et}_3\text{N}$  ( $6.27\ \mu\text{L}$ ,  $1.1\ \text{eq.}$ ) was added and the mixture was stirred for 30 min at rt protected from light. The solution was concentrated under vacuum and redissolved in DMF at  $15\ \text{mg/mL}$ . Several NGs, including control NGs lacking one of the monomers, were prepared. In addition, as observed for the unloaded NGs, the concentration of monomers/crosslinkers/drug in the organic phase (solvent) could be changed without significantly changing the size of the NGs. As a representative example, the synthesis of DOX-loaded NG containing all monomers and 10% of DOX is described. hPG acrylate ( $20\ \text{mg}$ ,  $\sim 1.6\ \mu\text{mol}$ ), AIBN ( $5\ \text{mg}$ ,  $30\ \mu\text{mol}$ ), DMDEA ( $32\ \text{mg}$ ,  $140\ \mu\text{mol}$ ), OEGA ( $28.34$

mg, 59.0  $\mu\text{mol}$ ), and BMADS (1.14 mg, 3.92  $\mu\text{mol}$ ) were dissolved in 0.5 mL of DMF containing DOX (7.5 mg). The organic mixture containing the drug, monomers, crosslinkers and initiator was immediately precipitated into 10 mL of a 50 mM phosphate buffer pH 8.0 solution which contained SDS (5  $\mu\text{M}$ ). The mixture was bubbled with Ar for 45 min and subsequently heated to 65  $^{\circ}\text{C}$  overnight. Then, the reaction flasks were opened to air to quench the reactions, and the resulting mixture was transferred into a centrifugal filter device (Amicon Ultra-15, 50 K) and washed 6 times with water, until no more red solution could be observed. Subsequently, the NGs were dialyzed for 12 h in PB pH 8 10 mM. After purification, NGs were run through a Sephadex G25 column (1 mL) to verify the absence of free drug. Mass recoveries varied in this case between 10 and 30%. The amount of the encapsulated DOX was estimated by UV-Vis spectroscopy in DMSO/H<sub>2</sub>O (9:1) ( $\lambda = 503 \text{ nm}$ ) after performing a calibration curve (Fig. S4). For the NGs containing all the monomers, the amount of DOX could also be calculated in water ( $\lambda = 488 \text{ nm}$ ,  $\epsilon = 11,500 \text{ L mol}^{-1} \text{ cm}^{-1}$ ). The loading weight percentage was calculated by the following equation, where  $W_{\text{DOX}}$  and  $W_{\text{NG}}$  stand for the encapsulated DOX weight and the weight of the NGs respectively:

$$\text{Loading (wt. \%)} = (W_{\text{DOX}} / W_{\text{DOX}} + W_{\text{NG}}) \times 100\%$$

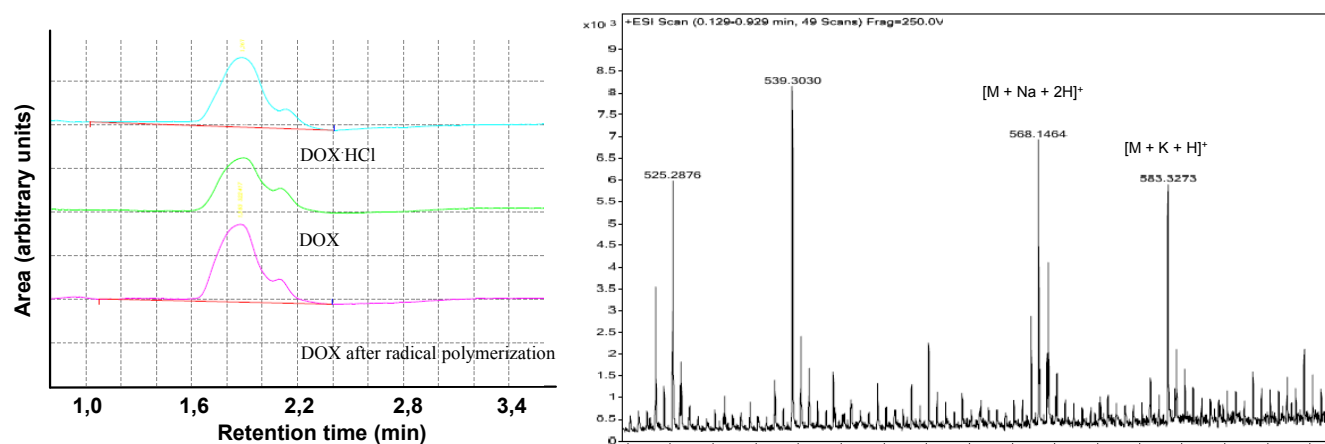


**Figure S4.** Calibration curve for DOX in DMSO/H<sub>2</sub>O (9:1), n = 3.

**HPLC characterization of DOX treated under free radical polymerization conditions.** The study of the stability of DOX after being submitted to NG synthetic conditions was carried out using a Knauer Smartline-HPLC system with an internal UV absorption detector ( $\lambda = 488 \text{ nm}$ ) and GeminyxSystem

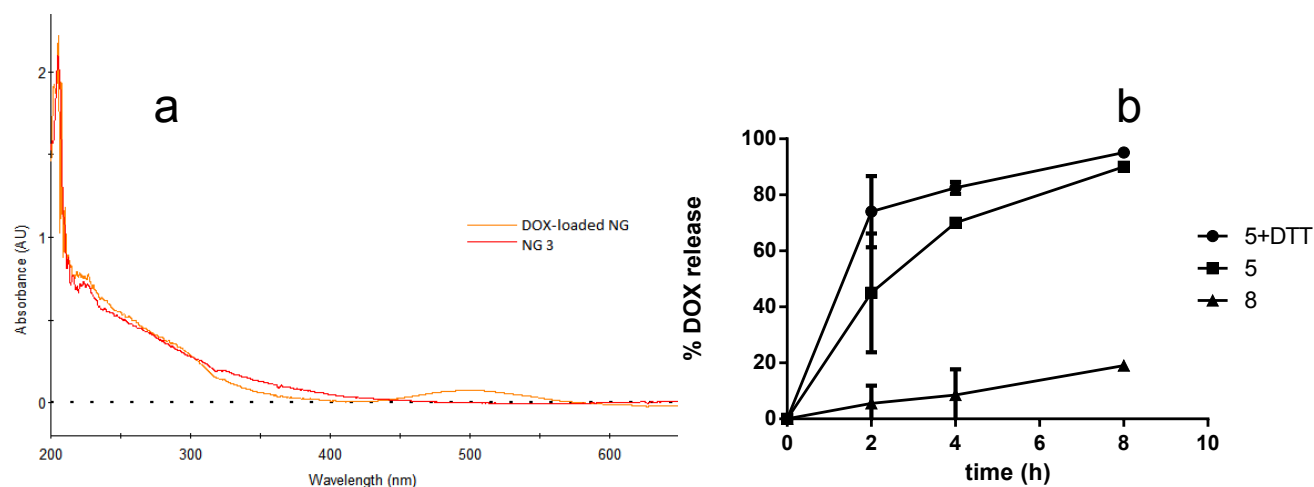


software. A Hypersil™ ODS C18 column (Thermo Scientific, 100 mm x 4.6 mm, particle Size: 5 μm) with a direct-connect guard column C18 was employed. Acetonitrile/water/trifluoroacetic acid (30:70:0.1) was used as the mobile phase at a flow rate of 1.0 mL/min under isocratic regime. Free DOX was submitted to NG formation conditions, purified by dialysis in water (100 Da MWCO membranes) and evaluated by HPLC. Untreated DOX·HCl, untreated DOX (i.e. after deprotonation with Et<sub>3</sub>N), and DOX treated under free radical polymerization conditions were dissolved in MeOH and analysed. Retention times and peaks shape were exactly the same for the 3 analytes. In addition, ESI-MS of DOX treated under free radical polymerization conditions was recorded.



**Figure S5.** Retention times of DOX·HCl, DOX and DOX after free radical polymerization (left) and ESI spectrum of DOX after free radical polymerization (right).

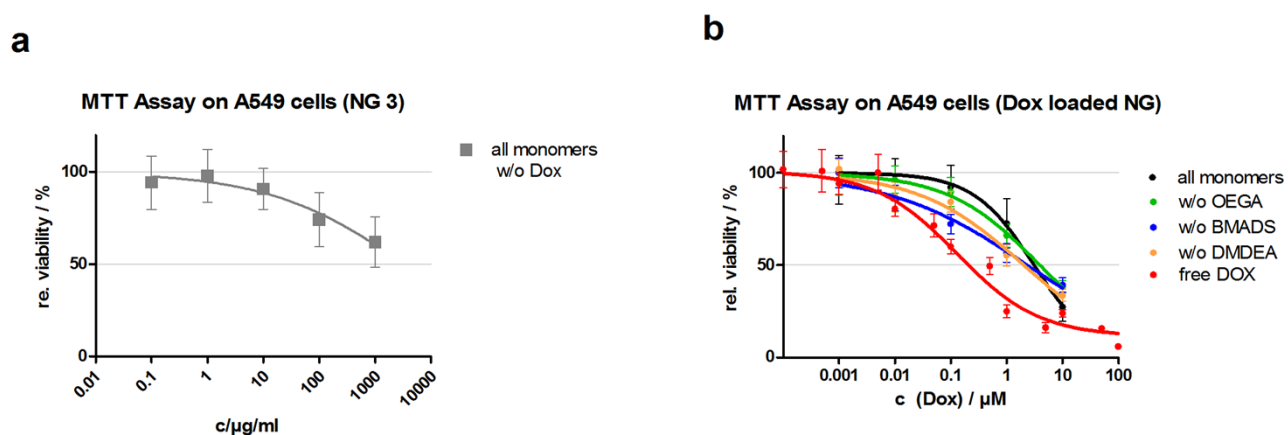
**In vitro DOX release studies.** NGs containing DOX were freeze-dried and redissolved in phosphate buffer 10 mM pH 8 or acetate buffer 10 mM pH 5. A solution of NGs in acetate buffer 10 mM pH 5 with DTT (20 mM) was also prepared. Aliquots (100 μL) were taken at different times and analysed by size exclusion chromatography using Sephadex G25 gel and water as running solvent. Free DOX was retained at the upper part of the column, while NGs could be collected and analysed by UV-Vis to determine DOX amounts ( $\lambda = 488 \text{ nm}$ ,  $\epsilon = 11500 \text{ L mol}^{-1} \text{ cm}^{-1}$ ).



**Figure S6.** (a) UV-Vis spectra of NG 3 and DOX-loaded NGs (all monomers) in H<sub>2</sub>O. (b) DOX release profiles from DOX-loaded NGs (all monomers) at pH 8, pH 5, and pH 5 + 20 mM DTT, n = 2.

**MTT assays.** A549 cells were routinely maintained in DMEM (Life Technologies) with 10% fetal calf serum (FCS, Biochrom AG) and 1% penicillin/streptomycin at 37 °C and 5% CO<sub>2</sub>. For MTT assay, 10000 cells/well were seeded into 96 well plates and grown overnight. On the next day, culture medium was discarded and replaced with 50 µL/well fresh medium. 50 µL of prediluted NGs (in medium) were added to the wells giving final concentrations as indicated in the graph (Fig. S7). The concentration of DOX-loaded NGs was calculated according to their loading with the drug and expressed as DOX equivalents (Fig. S7b). After 48 hours of incubation at 37 °C and 5% CO<sub>2</sub>, wells were washed twice with 200 µL PBS /well and 10 µL MTT solution (Sigma, 5 mg/mL stock solution in PBS) was added in 100 µL/well and again incubated for 4 h at 37 °C. The supernatant was then discarded and 100 µL/well of isopropanol with 0.04 M HCl was added to dissolve the dye crystals. After brief shaking, absorbance was measured at 570 nm in a Tecan Infinite 200 Pro microplate reader. All samples were incubated as triplicates on each 96-well plate. Average absorbance values were divided by those of untreated control cells to obtain the percentage of relative viabilities. Each experiment was repeated 3 times independently to calculate errors. The dose-response curve of free DOX shown in the graph (Fig. S7b), includes data from 25 independent assays we routinely run as a control in our lab. Differences in mean relative viabilities at 10 µM were analysed by One-way analysis of variance (ANOVA) followed by

Dunnett's Multiple Comparison post-test in GraphPad Prism software, results are summarized in Table S2.



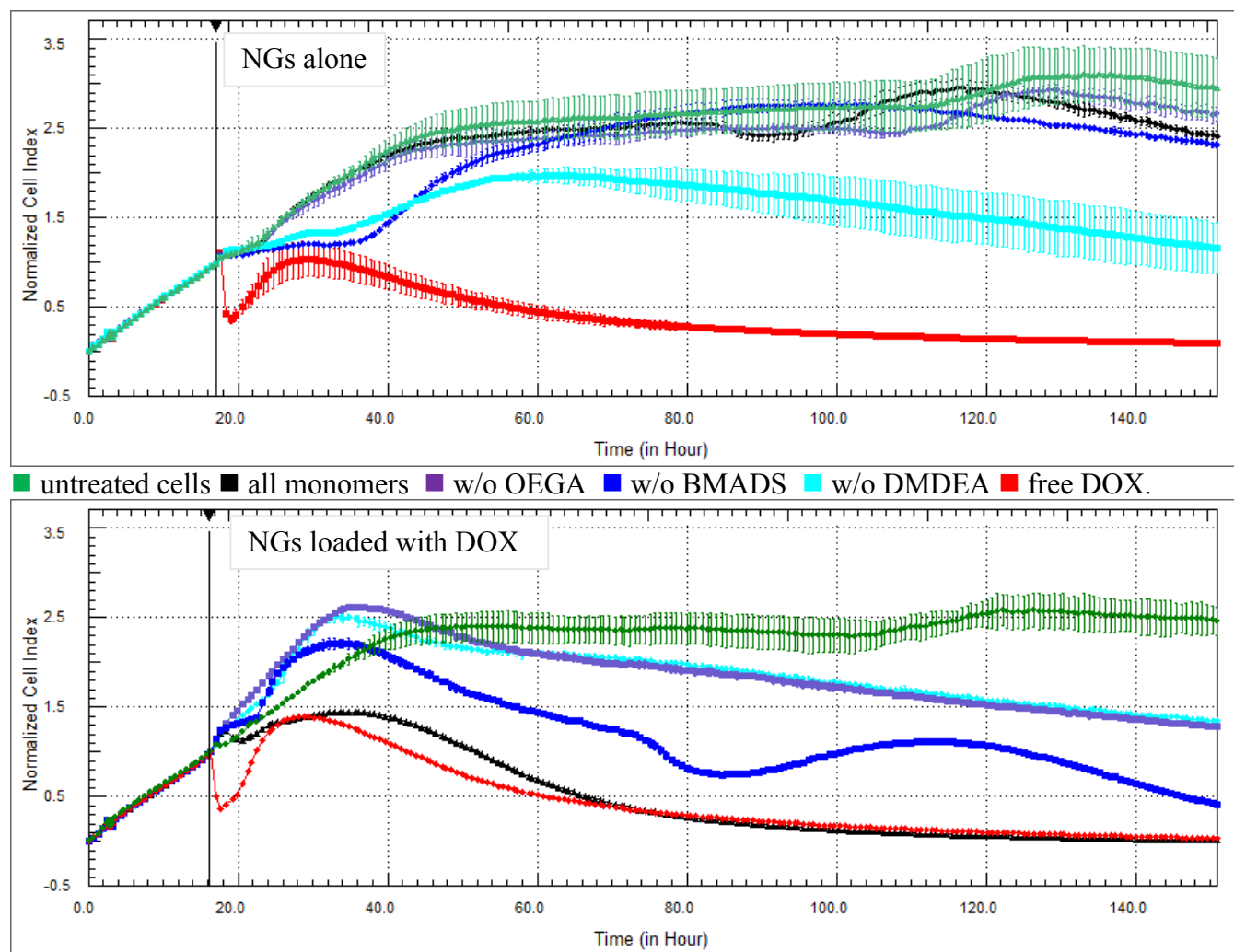
**Figure S7.** Viabilities of A549 cells after 48 h incubation with NG3 (a) or DOX-loaded NGs, n=3 (b) in comparison to free drug, as determined by MTT assay. Error bars are +/- SD, n=25 for DOX, n=3 for NGs.

**Table S2: Statistical analysis of relative viabilities at a fixed concentration of 10 µM**

One-way analysis of variance					
P value	0.0068				
P value summary	**				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	5				
F	6.716				
R squared	0.7287				
ANOVA Table					
	SS	df	MS		
Treatment (between columns)	936.7	4	234.2		
Residual (within columns)	348.7	10	34.87		
Total	1285	14			
Dunnett's Multiple Comparison Test					
	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
free DOX vs all monomers	-9.333	1.936	No	ns	-23.27 to 4.602
free DOX vs w/o OEGA	-20.33	4.217	Yes	**	-34.27 to -6.398
free DOX vs w/o BMADS	-21.33	4.425	Yes	**	-35.27 to -7.398
free DOX vs w/o DMDEA	-15.67	3.250	Yes	*	-29.60 to -1.731

**Real time cell analysis.** 10.000 A549 cells/well were seeded into an E-plate 16 (ACEA) and cell indices (CI) were recorded overnight in an xCELLigence® (ACEA Biosciences Inc.) real time cell analysis (RTCA) device. The next day, empty NGs or DOX-loaded NGs were added to the wells at a

final concentration of 100  $\mu\text{M}$  regarding DOX concentration, in duplicates. The concentration of unloaded NGs was the same as for the loaded ones. (All monomers: 0.54 mg/mL, w/o OEGA: 0.68 mg/mL, w/o BMADS: 0.68 mg/mL, w/o DMDEA: 0.54 mg/mL). Cell indexes were recorded for about 150 h. The experiment was repeated independently, confirming the reproducibility of the results shown in Fig. S8.



**Figure S8.** RTCA profiles of A549 cells incubated with different NGs (upper graph) and DOX-loaded NGs (lower graph) at the same concentrations (100  $\mu\text{M}$  DOX equivalents for the loaded NGs). The vertical line at  $t=16$  h marks the point where the NGs were added and cell indices were normalized.

Each of the experiments shown in Fig. S8 contained a control for the CI profiles of free DOX (red) on A549 cells as well as untreated cells (green). In this real time monitoring technique, the CI increases when more growth area is occupied by cells due to spreading, swelling and/or proliferation and

decreases when less area is occupied due to shrinkage and/or detachment. CI of untreated cells steadily increases until 40 h and then reaches a plateau. The small bump in the curve starting at about 110 h probably reflects morphological changes of the cell population which have been cultivated for a prolonged time at confluence. The profile for free DOX is characterized by a step-like decrease of CI directly at addition of the compound, which indicates a transient immediate physical effect on the cell morphology / attachment due to high concentrations of free DOX. This is followed by a slower increase in CI which forms a peak over the time of about 20 h. This peak can be regarded as the early cellular response to the drug. Afterwards, the CI decrease steadily indicating inhibition of proliferation and subsequently cell death and detachment. Comparing the profiles of the DOX-loaded and unloaded NGs, we see that the peak with which the cells respond immediately to DOX exposure are contained in the curves of the loaded NGs, but are absent from the curves of the unloaded NGs. Regarding unloaded NGs, the ones without DMDEA and without BMADS cause a plateau in the curves after the addition, for about 20 h duration. After this phase, the cells appear to proliferate again as if untreated in the case of the NG without BMADS (dark blue curve). The NGs without DMDEA (light blue curve) seem to affect cell proliferation because, after an increase in CI until 60 h, CI decreases slowly but steadily. A similar profile was seen in this NG when it is loaded with DOX, although again the peak of the early response is missing in the unloaded NGs. The unloaded NGs composed of all monomers (black curve) as well as the ones without OEGA (purple curve) appear to leave the cells widely unaffected. The slight bump that is seen in the curve of the untreated cells also occurs with these NGs, while it is shifted to an earlier time point with the NGs containing all monomers (about 90 h as compared to 110-120 h). Only the DOX-loaded NGs containing all monomers cause a similar profile than the free DOX. The DOX loaded NGs lacking OEGA or DMDEA are similar to each other, but both less effective in proliferation inhibition than the free drug and the NG containing all monomers.

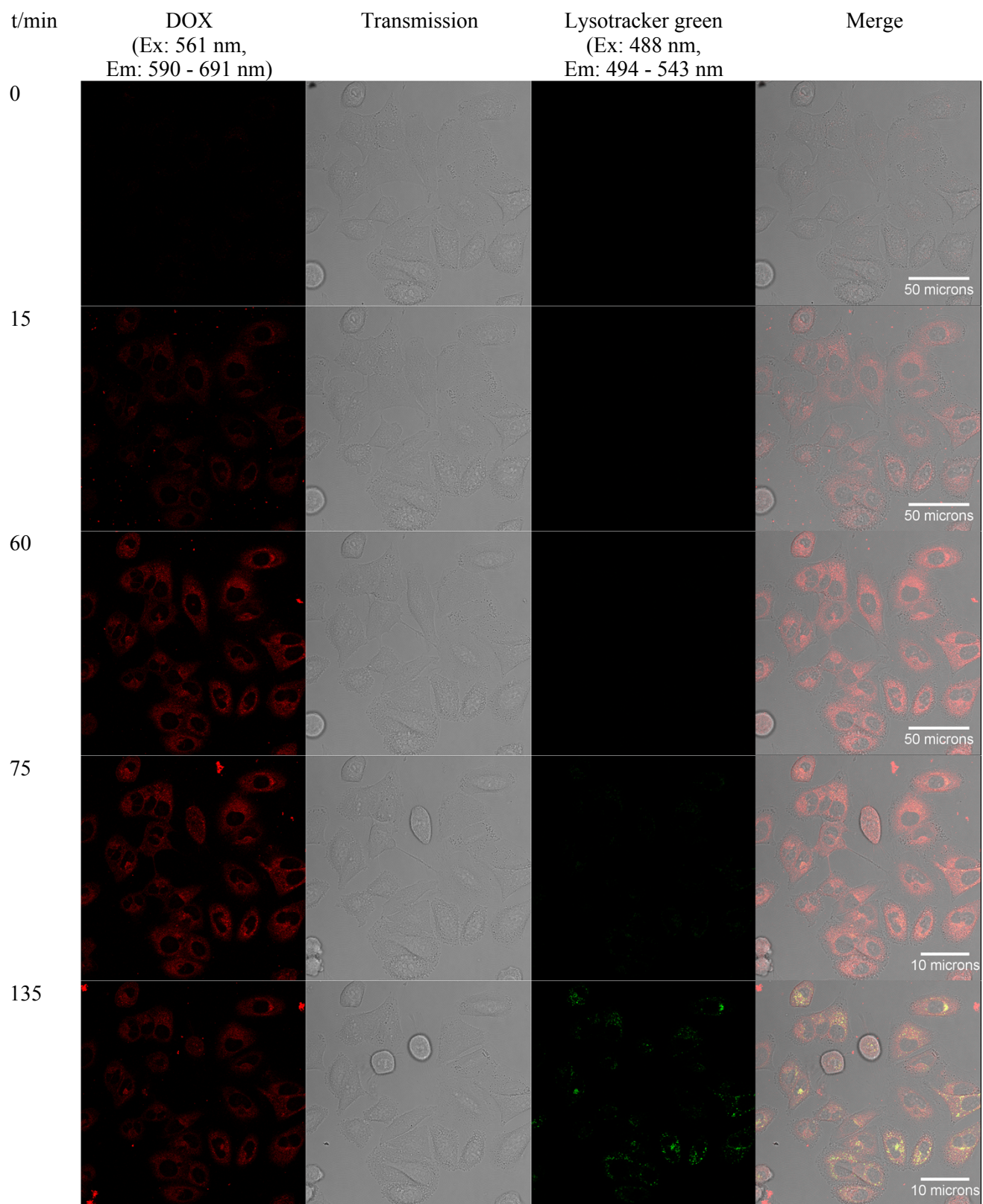
The DOX loaded NGs without BMADS appear to be slightly more effective than the ones without OEGA and DMDEA at first, but seem to lose their effect after 80 h. At this point, the curve shows a broad peak for the duration of about 50 h, which indicates that a population of cells proliferates again

before going to cell death. The reason for this (reproducible) profile has to be further investigated, but the absence of that re-growth from the profile of the unloaded NGs, as well as from all other curves, indicates that this profile is due to a different drug release mechanism.

**Cellular internalization studies.** Internalization of NGs into A549 cells was studied using a Leica SP8 confocal laser scanning microscope equipped with a live cell imaging chamber set to 37 °C and 5% CO<sub>2</sub>. 50000 A549 cells per mL were seeded into 35 mm  $\mu$ -dishes (ibidi) and grown in DMEM (Life Technologies) with 10% fetal calf serum (FCS, Biochrom AG) and 1% penicillin/streptomycin overnight at 37 °C and 5% CO<sub>2</sub>. The next day, the settings for the microscope fluorescence detectors were first set using another dish in which cells had been incubated with free DOX for 2 h, followed by incubation with LysoTracker green DND-26 (Life Technologies) for 10 min, to ensure that fluorescent signals are neither overlapping nor background. DOX-loaded NGs (all monomers, ~ 9% DOX) were added to a final concentration of 2  $\mu$ M (DOX) and images were acquired every 15 s for 1h using LASX Software (Leica). The medium was replaced with fresh medium containing LysoTracker green after 1 h 15 min, and image acquisition was continued for another hour. Representative images at 5 different time points were selected and are shown in Fig. S9.

An increase in DOX fluorescence in the cells could be seen over time, locating in the perinuclear region and punctate structures. After addition of LysoTracker green, which accumulates in acidic compartments of the cells, an overlap of the red and green fluorescence signals in those compartments could be observed, leading to yellow color in the merged images.





**Figure S9.** Confocal laser scanning images of A549 cells incubated with DOX-loaded NG 3 and Lysotracker green (added after 1 h 20 min) showing cellular uptake and accumulation in acidic compartments of the cells over time.

## References

1. D. Steinhilber, S. Seiffert, J. A. Heyman, F. Paulus, D. A. Weitz and R. Haag, *Biomaterials*, 2011, **32**, 1311-1316.
2. Z.-Y. Qiao, F.-S. Du, R. Zhang, D.-H. Liang and Z.-C. Li, *Macromolecules*, 2010, **43**, 6485-6494.