SUPPLEMENTARY MATERIAL

Smart nanoformulation based on stimuli-responsive nanogels and curcumin: Promising therapy against colon cancer

Lizbeth A. Manzanares-Guevara[1], Angel Licea-Claverie[1,*], Irasema Oroz-Parra [2], Johanna Bernaldez-Sarabia[3], Fernando Diaz-Castillo[3], and Alexei F. Licea-Navarro[3].

[1]Centro de Graduados e Investigación en Química, Instituto Tecnológico de Tijuana, Tijuana, Baja California, México
[2]Facultad de Ciencias Marinas, Universidad Autónoma de Baja California, Ensenada, Baja California, México.
[3]Departamento de Innovación Biomédica, Centro de Investigación Científica y de Educación Superior de Ensenada (CICESE), Ensenada, Baja California, México.

Correspondence to: Prof. Dr. Angel Licea-Claverie

Centro de Graduados e Investigación en Química, Instituto Tecnológico de Tijuana. A. P. 1166.

C.P. 22000, Tijuana, Baja California, México

Phone: 52-664-6233772// Fax: 52-664-6234043// E-mail: aliceac@tectijuana.mx

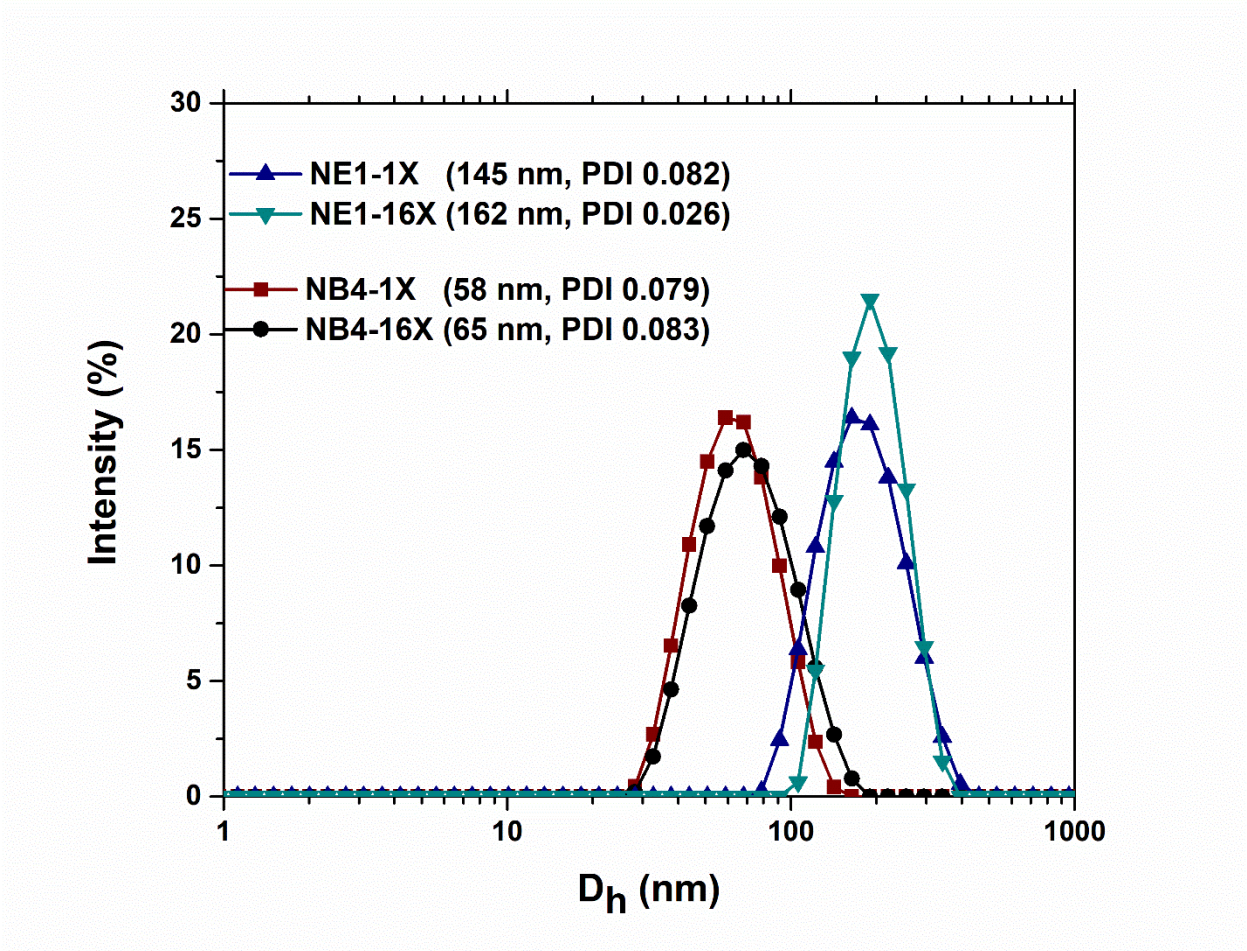

Figure S1. Particle size distribution of PDEAEM-*core*-PEG-*shell* nanogels crosslinked with EGDMA (NE1) and crosslinked with BAC (NB4): Comparison of nanogels prepared in small scale (1X=50 mL) and in large scale (16X=800mL).

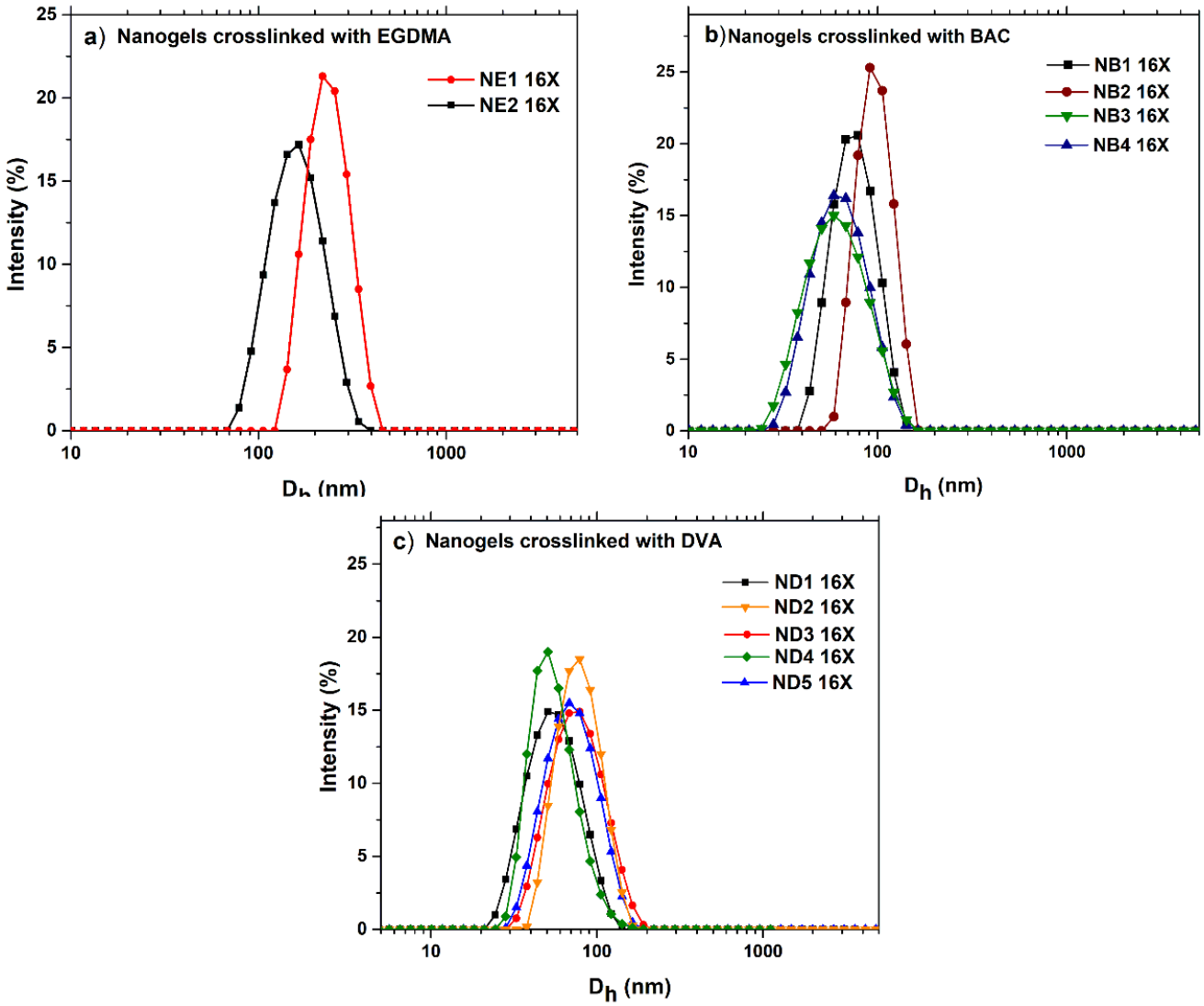

Figure S2. Particle size distribution of PDEAEM-*core*-PEG2000-*shell* nanogels: a) crosslinked with EGDMA, b) crosslinked with BAC, c) crosslinked with DVA.

CHARACTERIZATION OF NANOGELS USING NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Given the fact that the amount of crosslinker used in the nanogels synthesis is less than 3 mol% of the DEAEM content and the DEAEM content in the feed is less than 70 % of the total mass content in the nanogels, then by ^{1}H-NMR it is hard to recognize the crosslinker.

However the presence of PDEAEM, PEGMA and its content is clearly seen. The description of all signals and the composition determination is described for one example, the PDEAEM-*core*-PEGMA-*shell* nanogel crosslinked with DVA (ND3), spectrum in Figure S3:

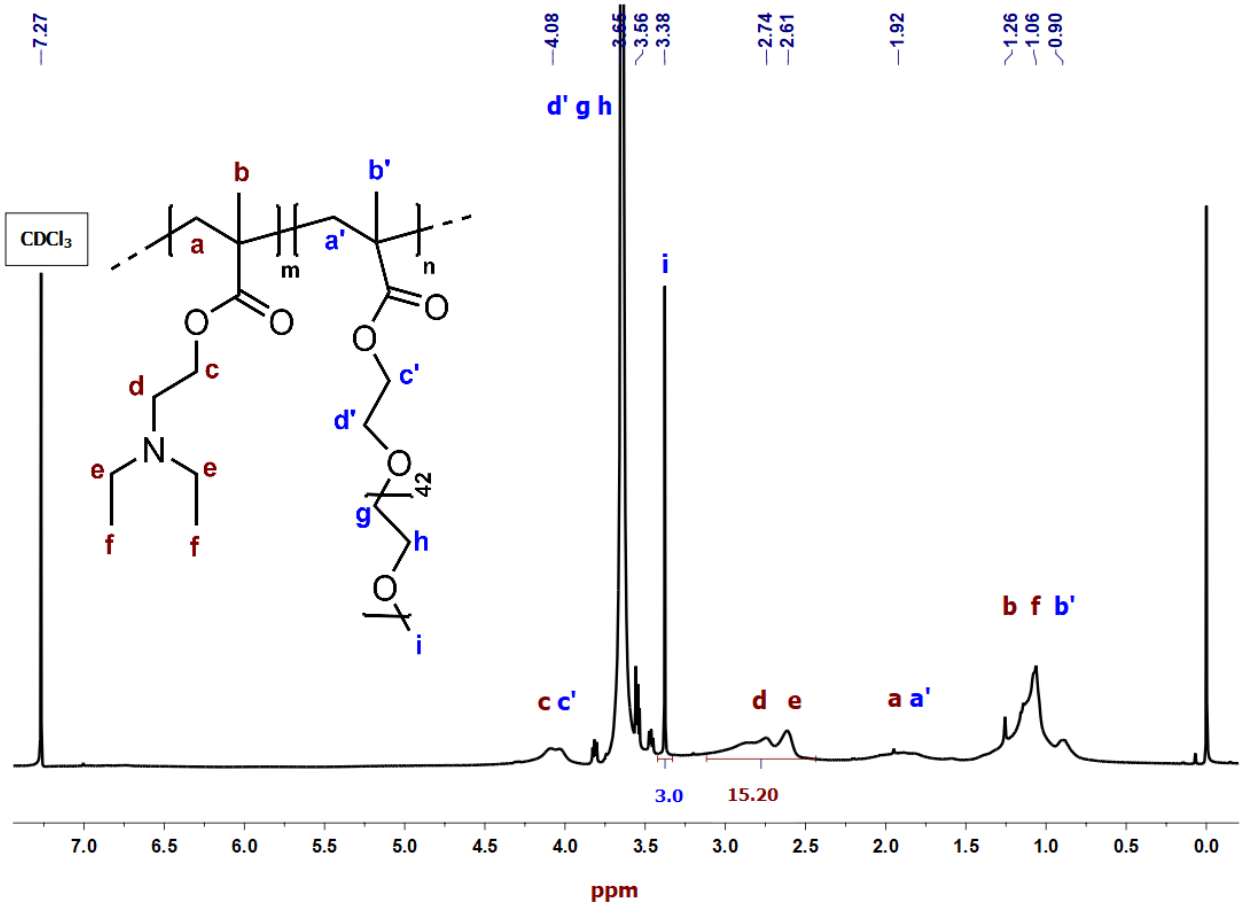

Figure S3. ^{1}H-NMR spectrum in $CDCl_3$ of a PDEAEM-*core*-PEG-*shell* nanogel crosslinked with DVA (ND4).

The signals between 0.9 and 1.4 ppm are assigned to the -CH_3 groups of the methacrylates (b=DEAEM and b'=PEGMA) and also to the hydrogens of the methyl-groups (f) of PDEAEM. The overlapping signal between 1.5 and 2.2 ppm corresponds to the -CH_2- groups of the polymer backbone (a, a´); the signals between 2.5 and 3 ppm corresponds to the six hydrogens (d, e) of the methylene groups (-N-CH_2-) adjacent to the nitrogen of DEAEM; the signal at 3.38 ppm corresponds to the methyl hydrogens (i) of the methoxy end-group of PEGMA; the strong signal at 3.7 pm corresponds to the -CH_2-O (d', g. h) of PEGMA; the signal at 4.1 ppm corresponds to two hydrogens (c) of the –CH_2-O of the ester group of DEAEM and two hydrogens(c') of the –CH_2-O of the ester group of PEGMA. The peak at 7.2 ppm corresponds to the deuterated solvent ($CDCl_3$). The composition was calculated by integration of signals at chemical shifts of 3.38 ppm (i) (3H) in the end group of the PEG side-chains and the integration of the signals between 2.5 and 3 ppm (d, e) (6H) of mehtylenes attached to the amine group of DEAEM as follows:

PDEAEM:PEGMA (molar ratio) by ^{1}H-NMR
PDEAEM: 6H =15.20 (integration); therefore, 1H = 2.53
PEGMA: 3H = 3 (integration); therefore, 1H=1.0
PDEAEM (1H)+PEGMA(1H)=3.53
PDEAEM content (mol%) =2.53/3.53=0.716 (molar), **71.6mol%**
PEGMA content (mol%) =1.0/3.53=0.283 (molar), **28.3 mol%**

PDEAEM:PEGMA (weight ratio) by ^{1}H-NMR
PDEAEM (weight)=(0.716 mol)(185.27 g/mol)=132.65 g
PEGMA (weight)=(0.283 mol)(2000 g/mol)=566.4 g
PDEAEM (weight) +PEGMA (weight)=699 g
PDEAEM(weight%)=132.65 g/699 g =0.19(wt), **19 wt%**
PEGMA(weight%)=566.4 g/699 g=0.81(wt), **81 wt%**

Following the same methodology, the composition of all nanogels presented in Table 1 of the manuscript was determined, Representative spectra are shown in **Figures S3-S6**, the spectra of all the nanogels shown in **Table 1** are available upon request.

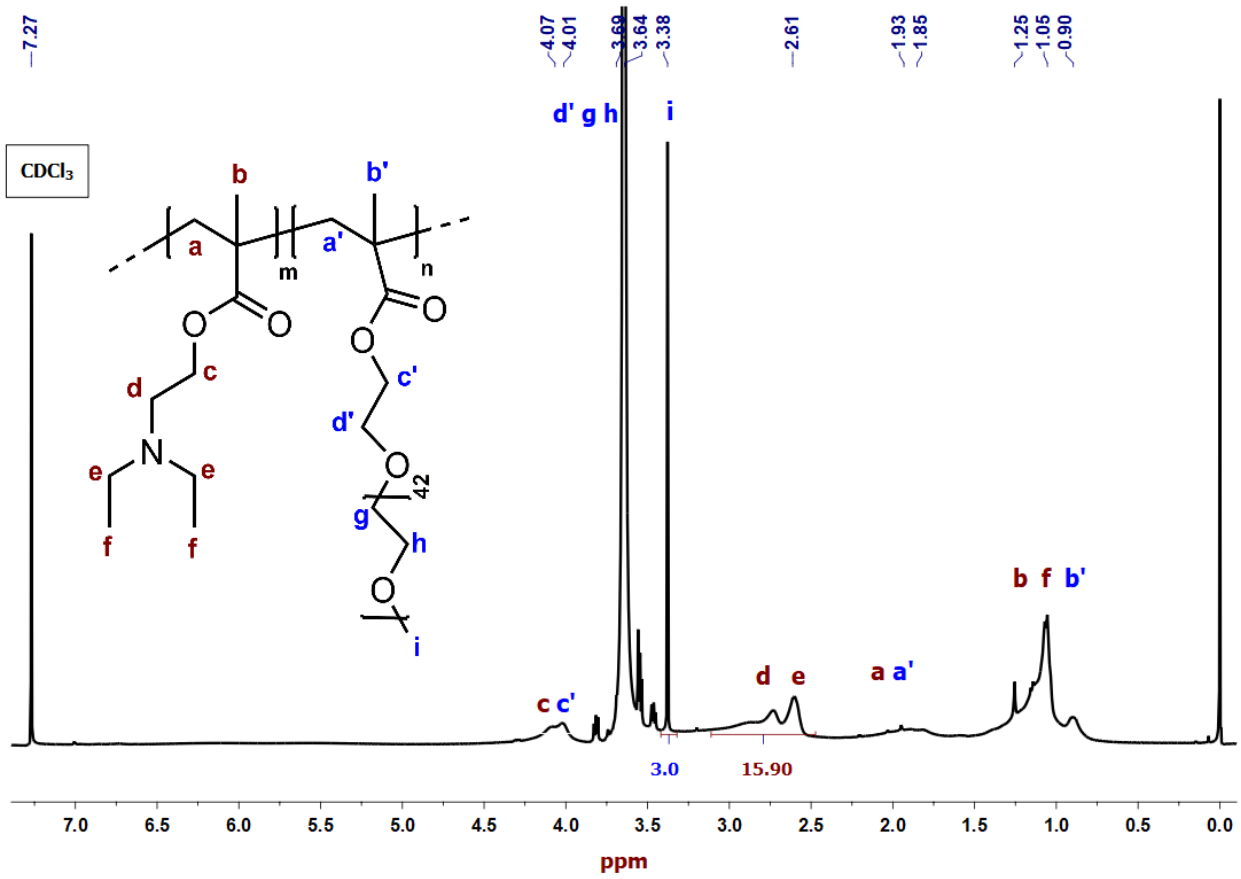

Figure S4. ^{1}H-NMR spectrum in $CDCl_3$ of a PDEAEM-*core*-PEG-*shell* nanogel crosslinked with BAC (NB4).

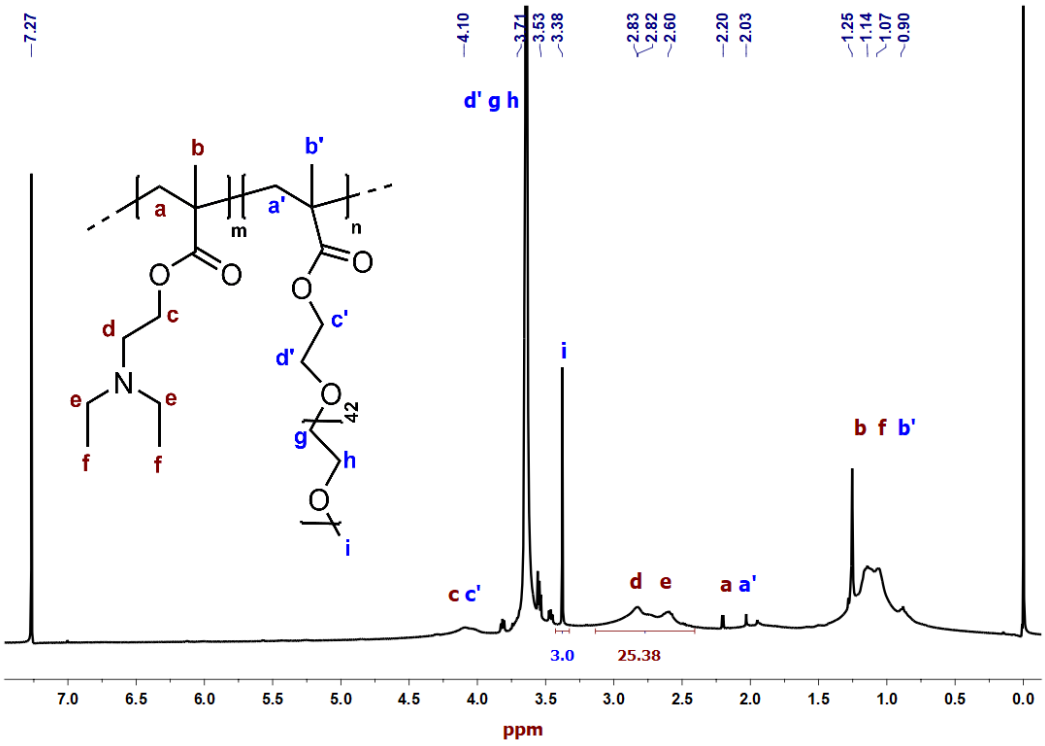

Figure S5. ^{1}H-NMR spectrum in $CDCl_3$ of a PDEAEM-*core*-PEG-*shell* nanogel crosslinked with EGDMA (NE2).

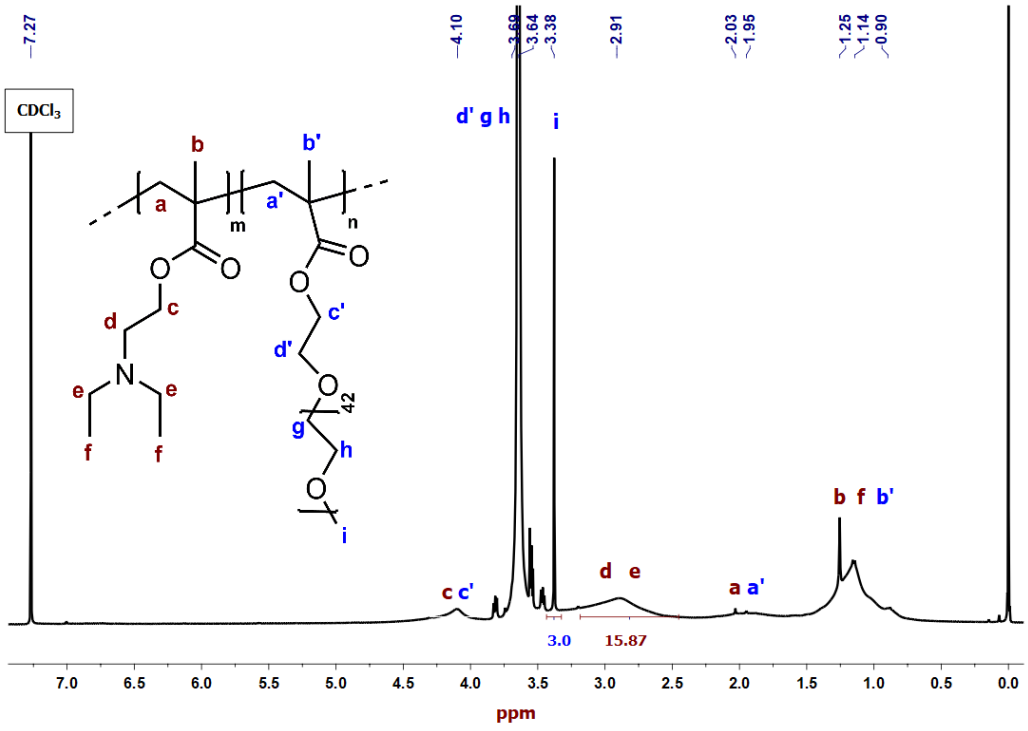

Figure S6. ^{1}H-NMR spectrum in $CDCl_3$ of a PDEAEM-*core*-PEG-*shell* nanogel crosslinked with FDAC (NF1).

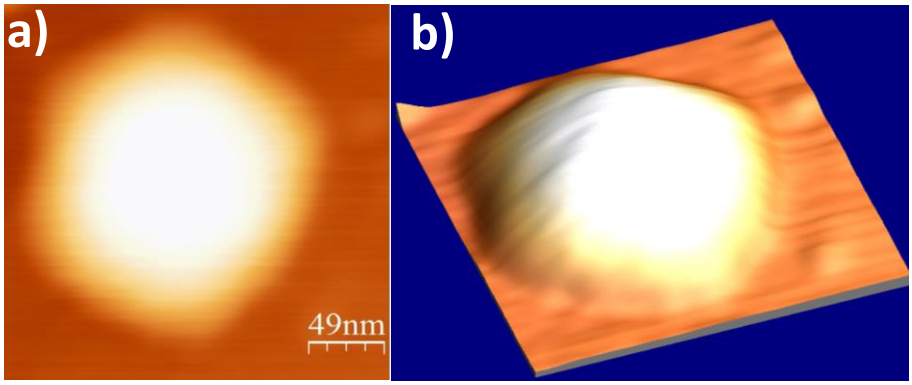

Figure S7. AFM images of nanogels over mica surface: a) 2D topography image of one PDEAEM-*core*-PEG-*shell* nanogel crosslinked with EGDMA (NE1) D_h = 142 nm; b) 3D topography image of one nanogel (NE1).

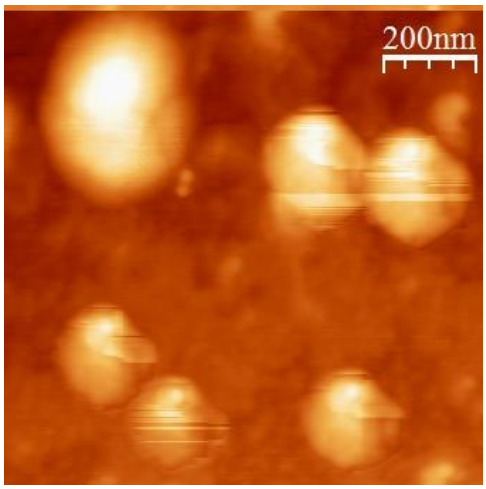

Figure S8. AFM-2D topography image of PDEAEM-*core*-PEG-*shell* nanogels crosslinked with DVA (ND2), over mica surface.

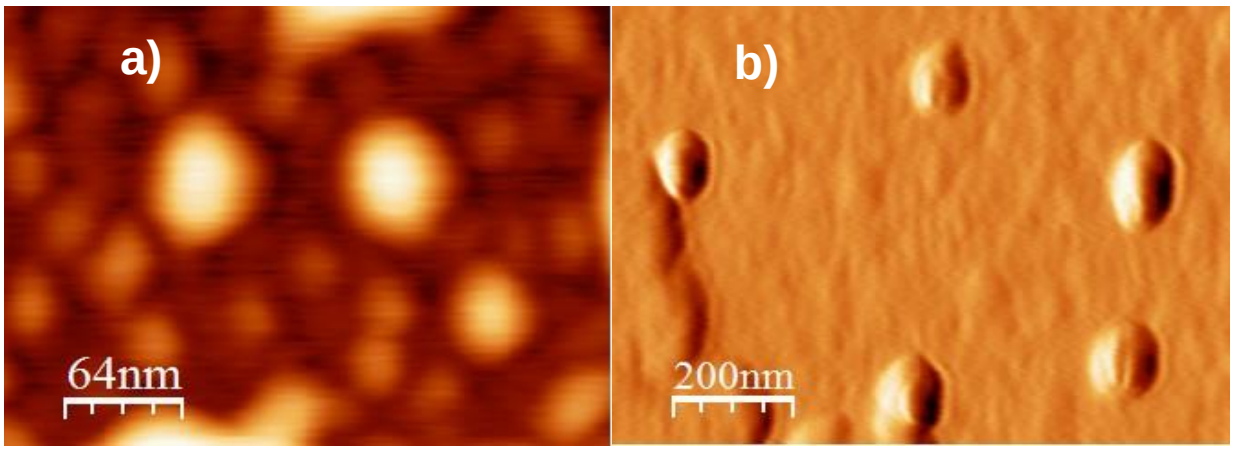

Figure S9. AFM-2D topography images of PDEAEM-*core*-PEG-*shell* nanogels crosslinked with BAC over mica surface: a) NB1 and b) NB2.

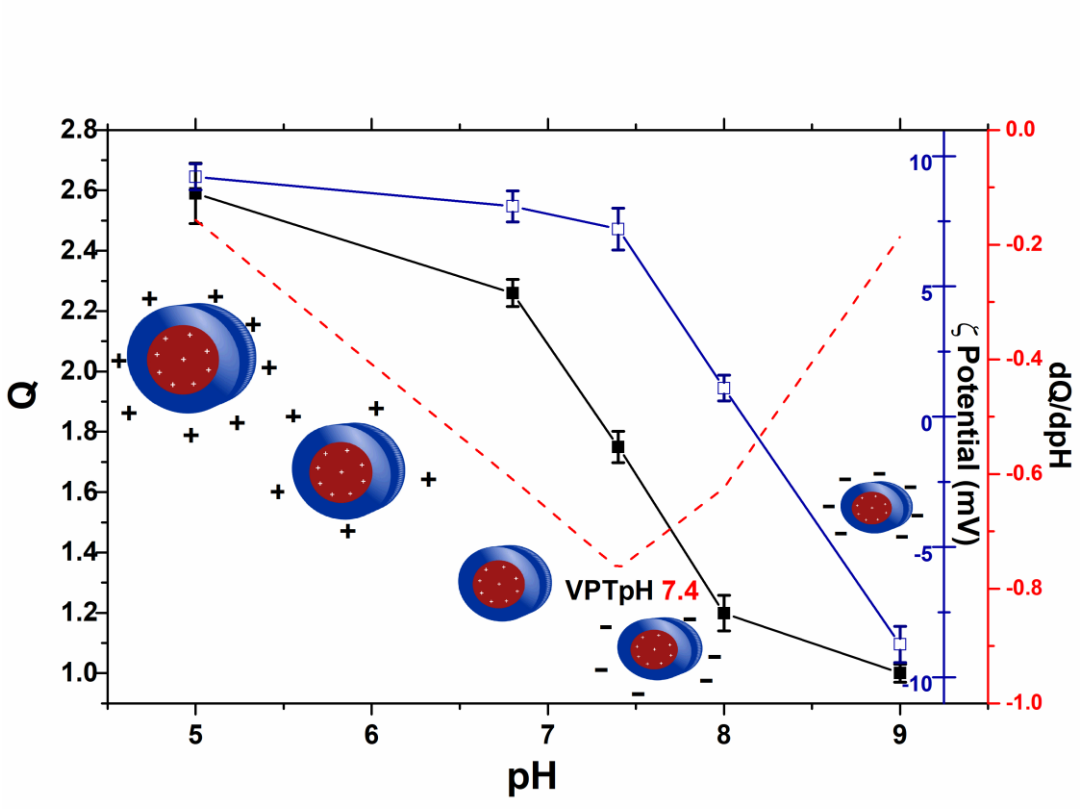

Figure S10. Responsive behavior of PDEAEM-*core*-PEG-*shell* nanogels (NE1, crosslinked with EGDMA): D_h and ζ potential as a function of pH.

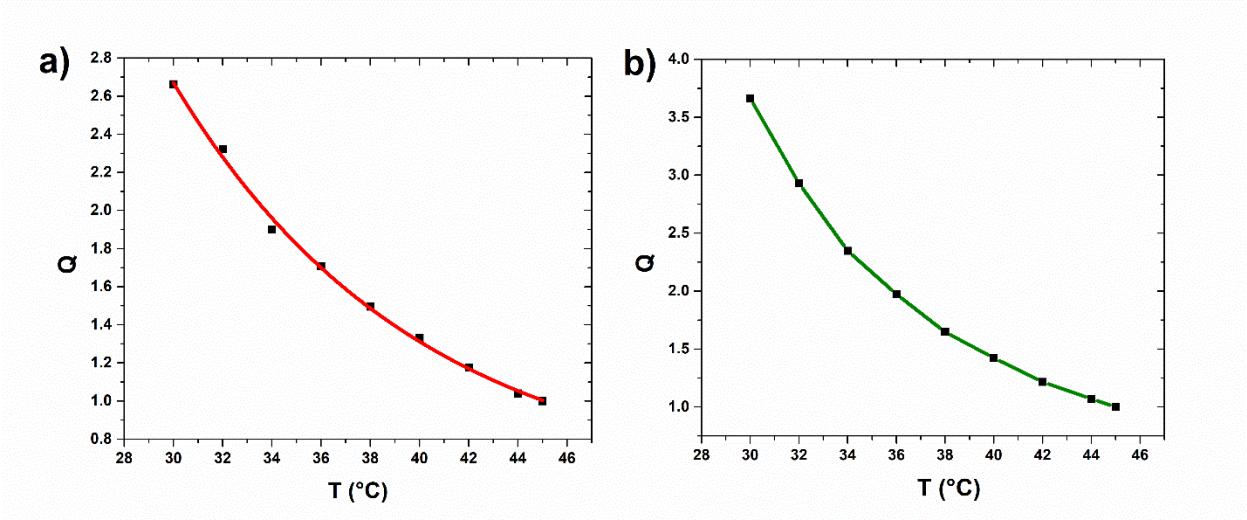

Figure S11. Responsive behavior of PDEAEM-*core*-PEG-*shell* nanogels, D_h as a function of temperature at pH 7.4: a) ND3, crosslinked with DVA, b) NB1, crosslinked with BAC.

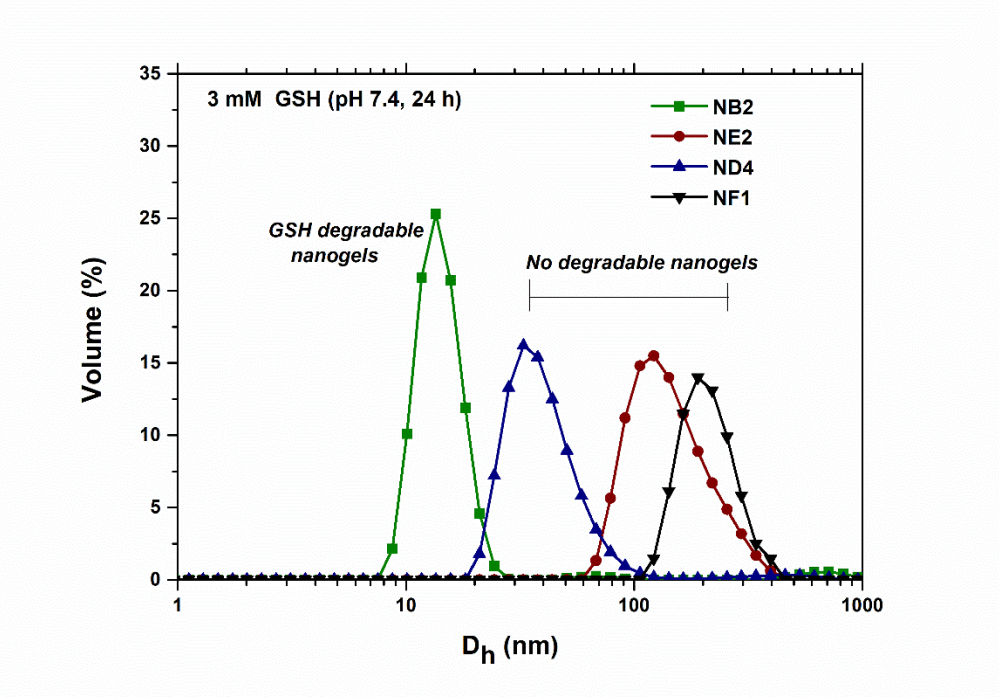

Figure S12. Degradation studies of PDEAEM-*core*-PEG-*shell* nanogels prepared using different crosslinkers: Size distributions of nanogels by DLS after 24 h of contact with 3 mM GSH at pH 7.4.

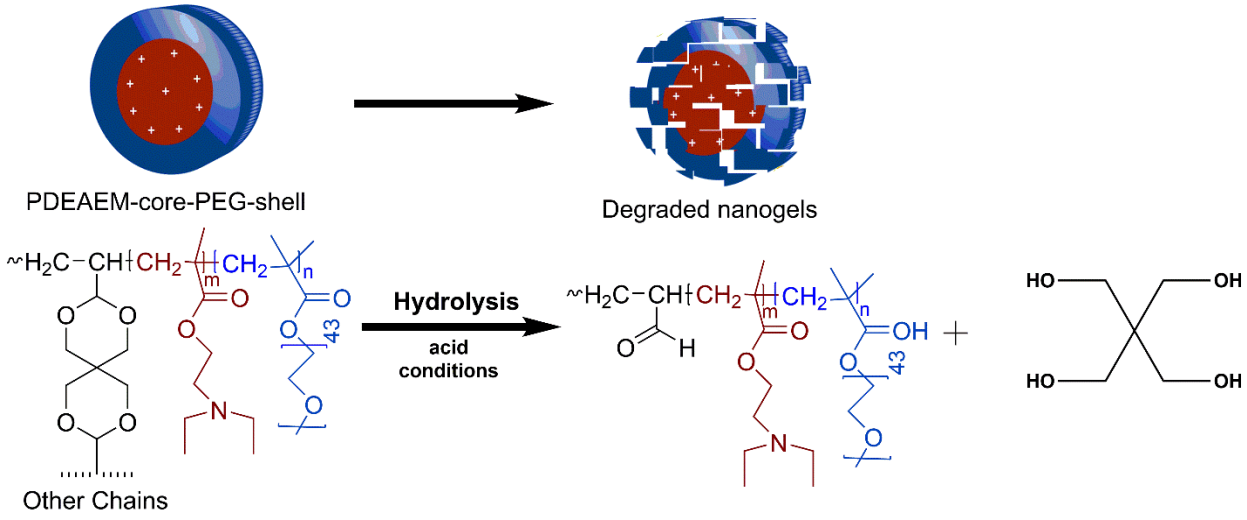

Figure S13. Scheme of acid-degradation of pH-responsive PDEAEM-*core*-PEG-*shell* nanogels crosslinked with DVA (ND3).

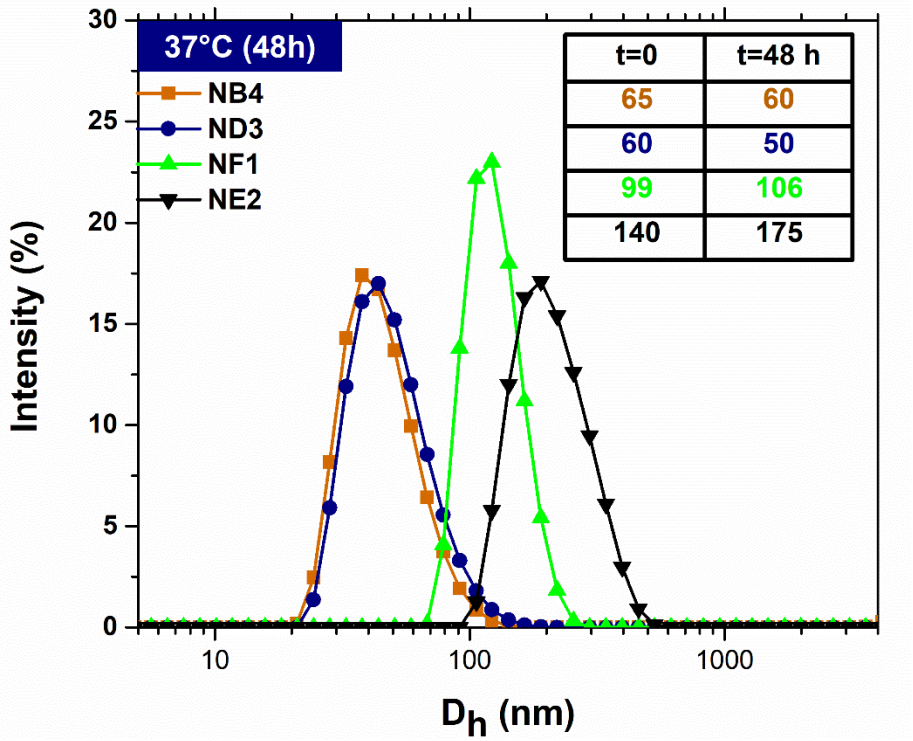

Figure S14. Stability studies of PDEAEM-*core*-PEG-*shell* nanogels prepared using different crosslinkers (NE2, NB3, ND4 and NF1). The evolution of the hydrodynamic diameter (D_h) was monitored by DLS at pH 7.4 (37 °C) up to 48 h.

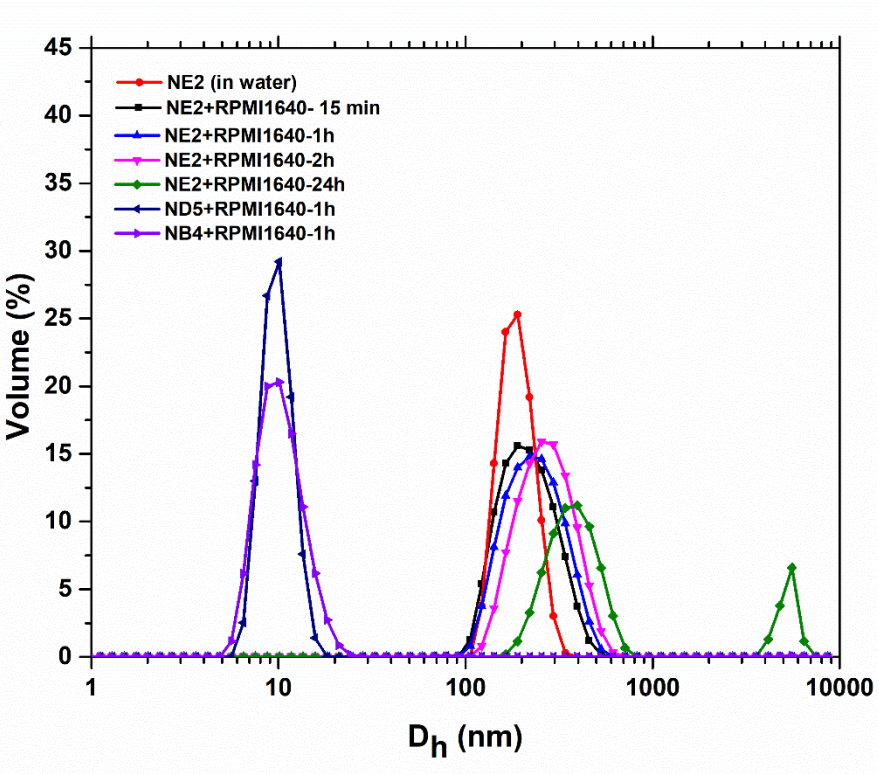

Figure S15. Stability for PDEAEM-*core*-PEG-*shell* nanogels at 37°C in biological mimicking media (Concentration 25 µg/mL). Cell culture medium (RPMI-1640) supplemented with 10% FBS.

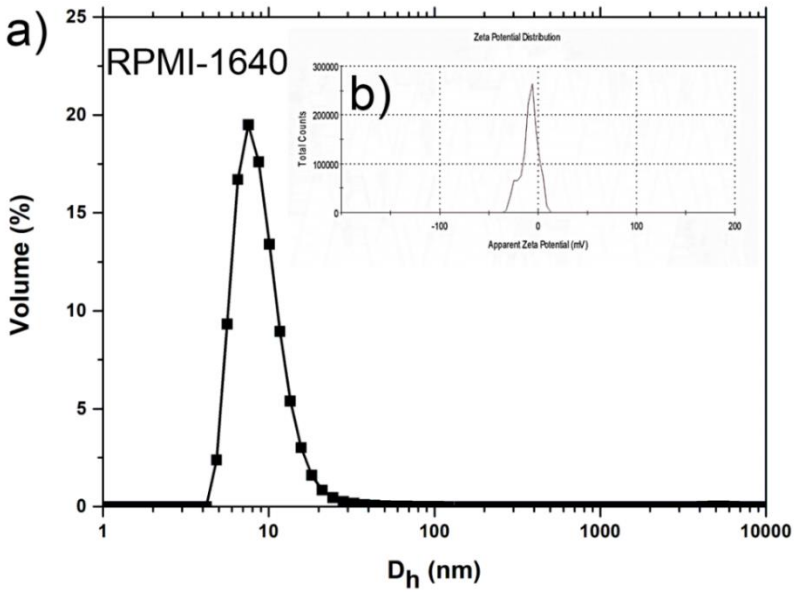

Figure S16. RPMI-1640 supplemented culture medium: a)Size distribution and b)zeta potential.

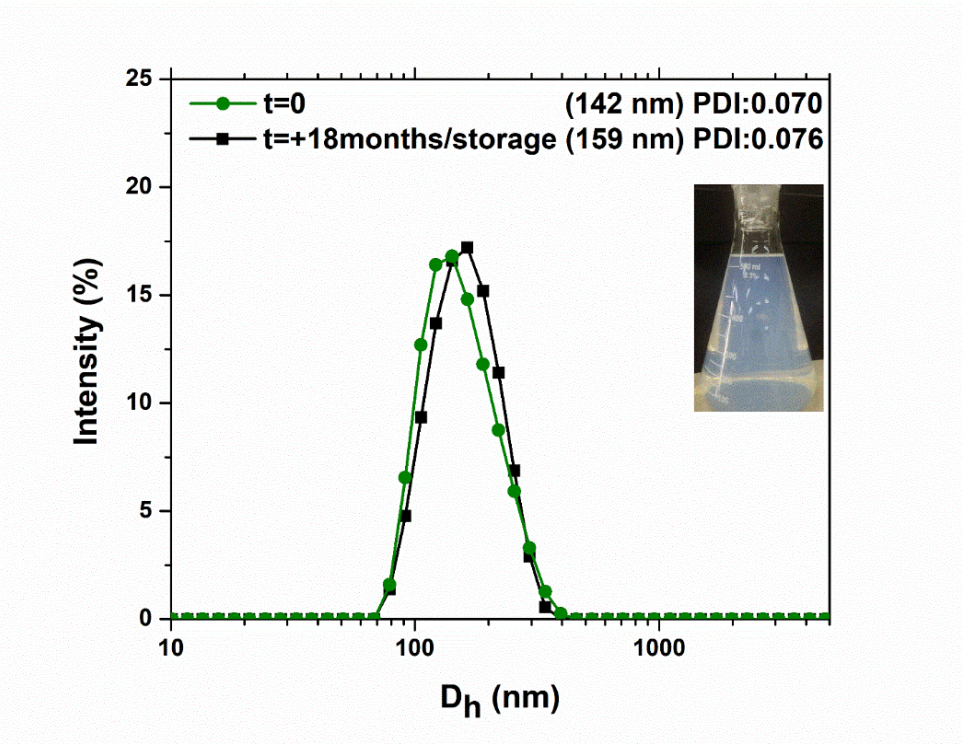

Figure S17. Stability at storage conditions (25 ^{o}C) of PDEAEM-*core*-PEG-*shell* nanogels crosslinked with EGDMA (NE2). (Photograph taken by the authors of the manuscript).

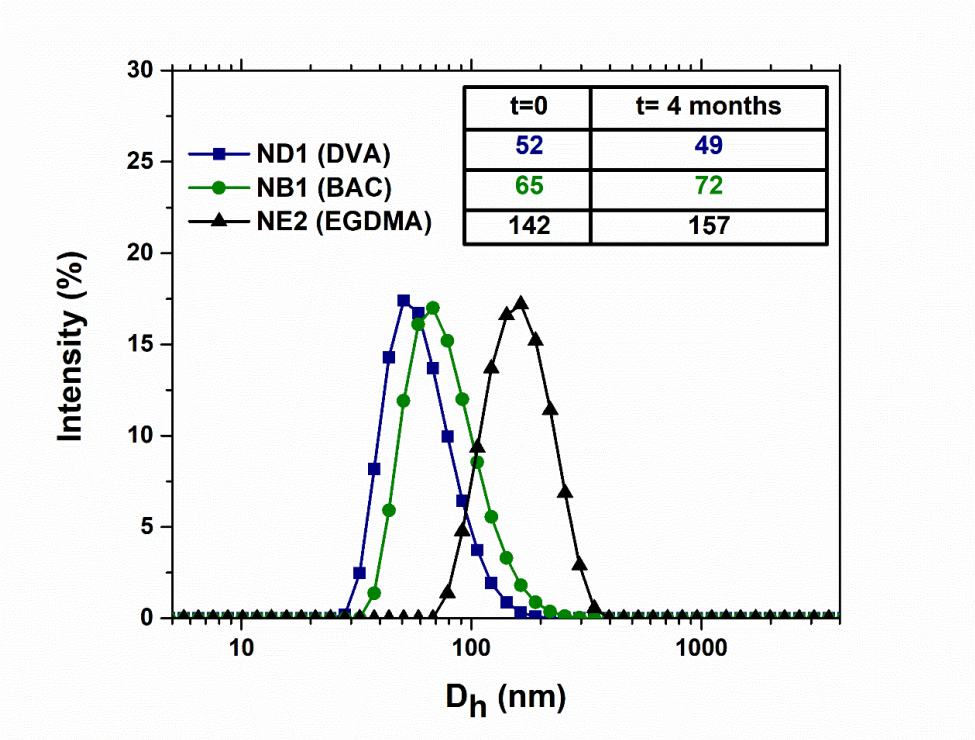

Figure S18. Stability at storage conditions (25 ^{o}C) of PDEAEM-*core*-PEG-*shell* nanogels crosslinked with DVA, BAC and EGDMA.

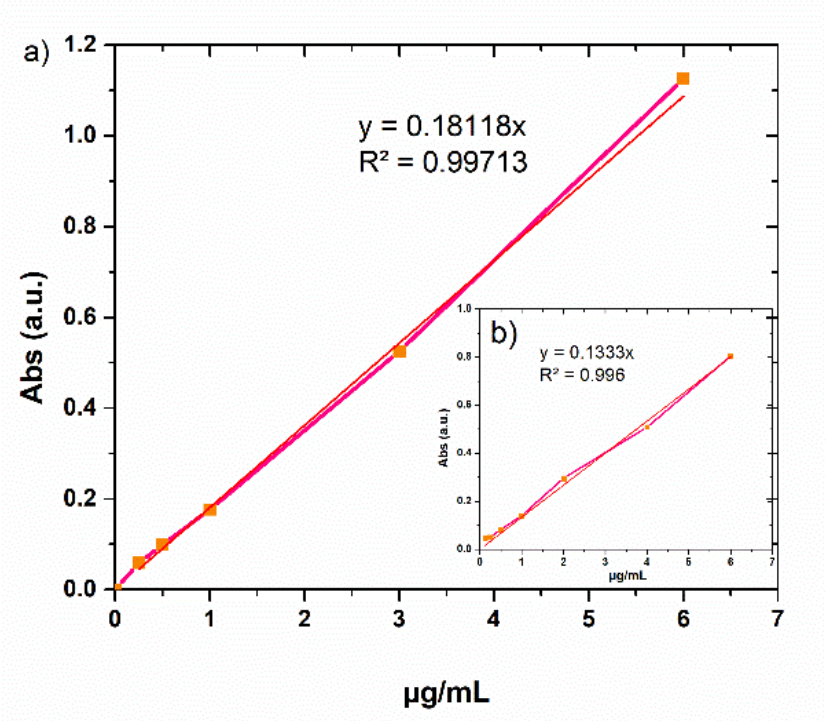

Figure S19. UV-vis **c**alibration curves for Curcumin at 427 nm: a) in EtOH and b) in PBS+Tween®80 (0.5 %).

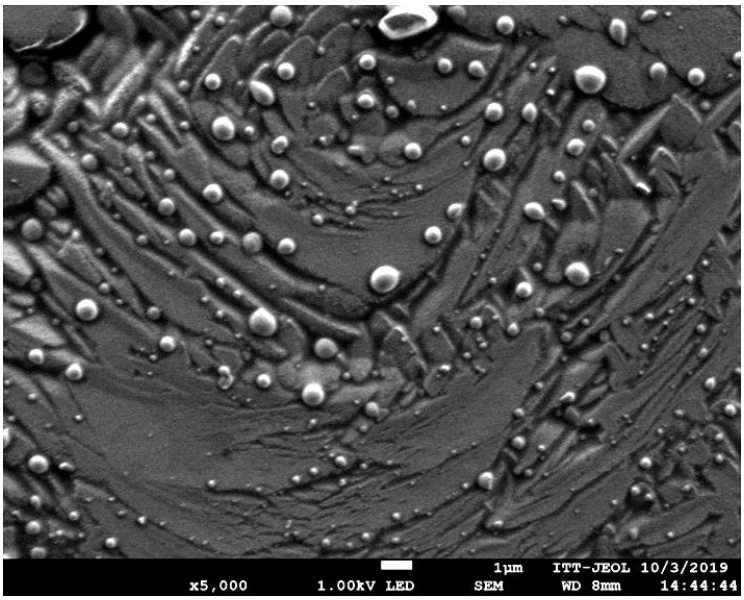

Figure S20. FESEM image of CUR-loaded PDEAEM-*core*-PEG-*shell* nanogels crosslinked with DVA (ND3).

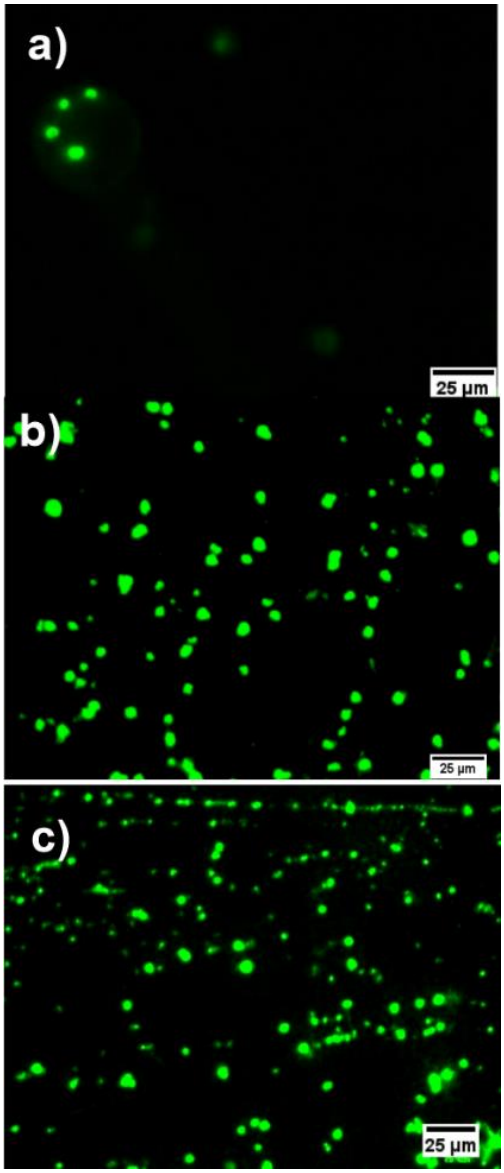

Figure S21. Fluorescence images of clusters of CUR-loaded in PDEAEM-*core*-PEG-*shell* nanogels: a) NE2 (crosslinked with EGDMA), b) ND3 (crosslinked with DVA), c) NB4 (crosslinked with BAC).

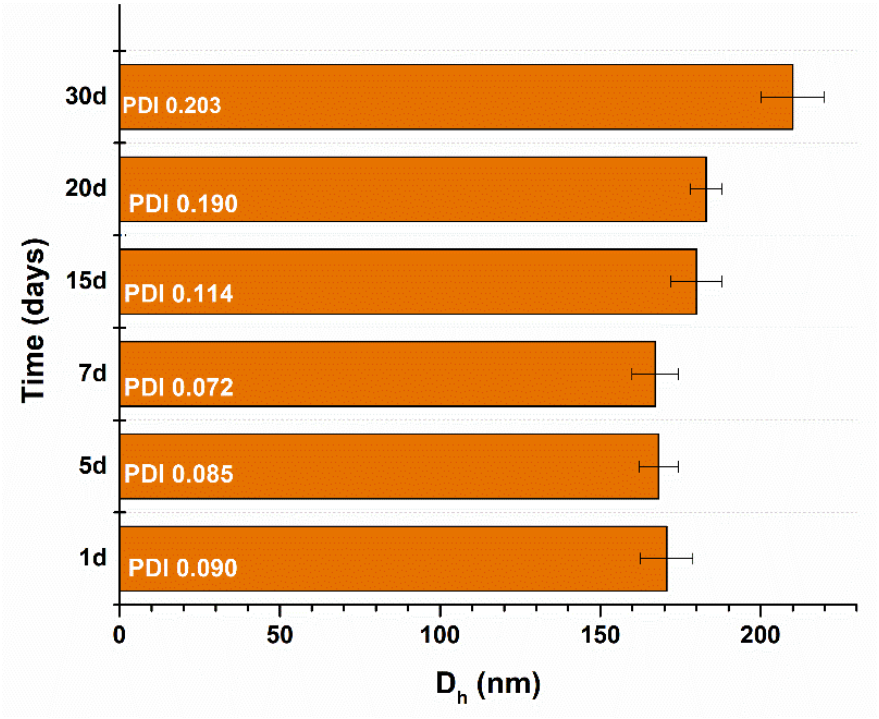

Figure S22. Stability of CUR-loaded PDEAEM-*core*-PEG-*shell* nanogels (NE2) at 37 ºC in the dark.

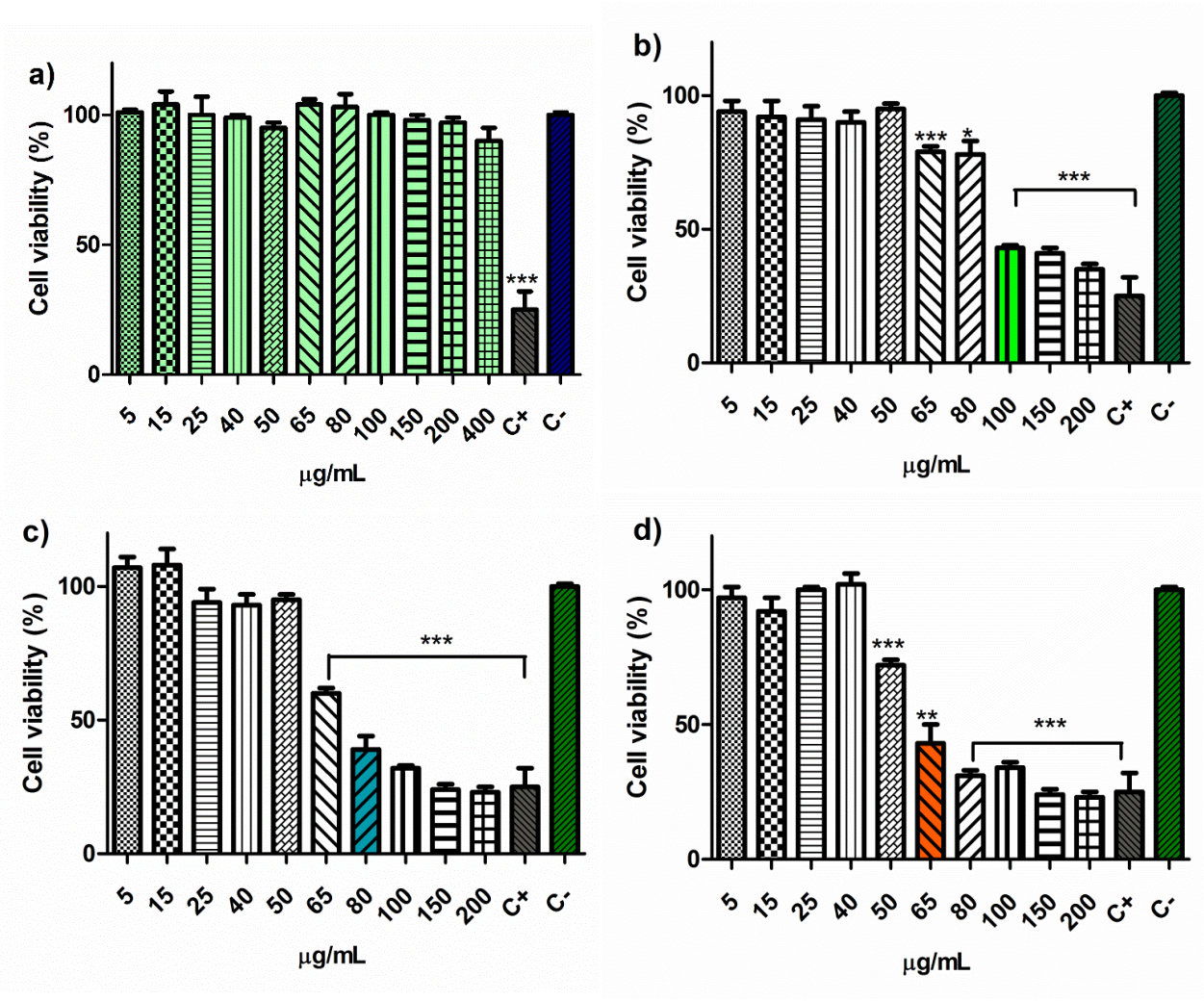

Figure S23. Doses-response assay for empty nanogels into human colon-cancer cell line (HCT-116): a) NE2 (EGDMA crosslinked), b) NF1 (FDAC crosslinked), c) ND3 (DVA crosslinked), d) NB4 (BAC crosslinked),. The cell viability (%) of cells is expressed as functions of untreated cells (C-). The results represent the average ± SEM of triplicates. Positive control (C+) 5 % DMSO. *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 vs C- (unpaired t-Student's test).

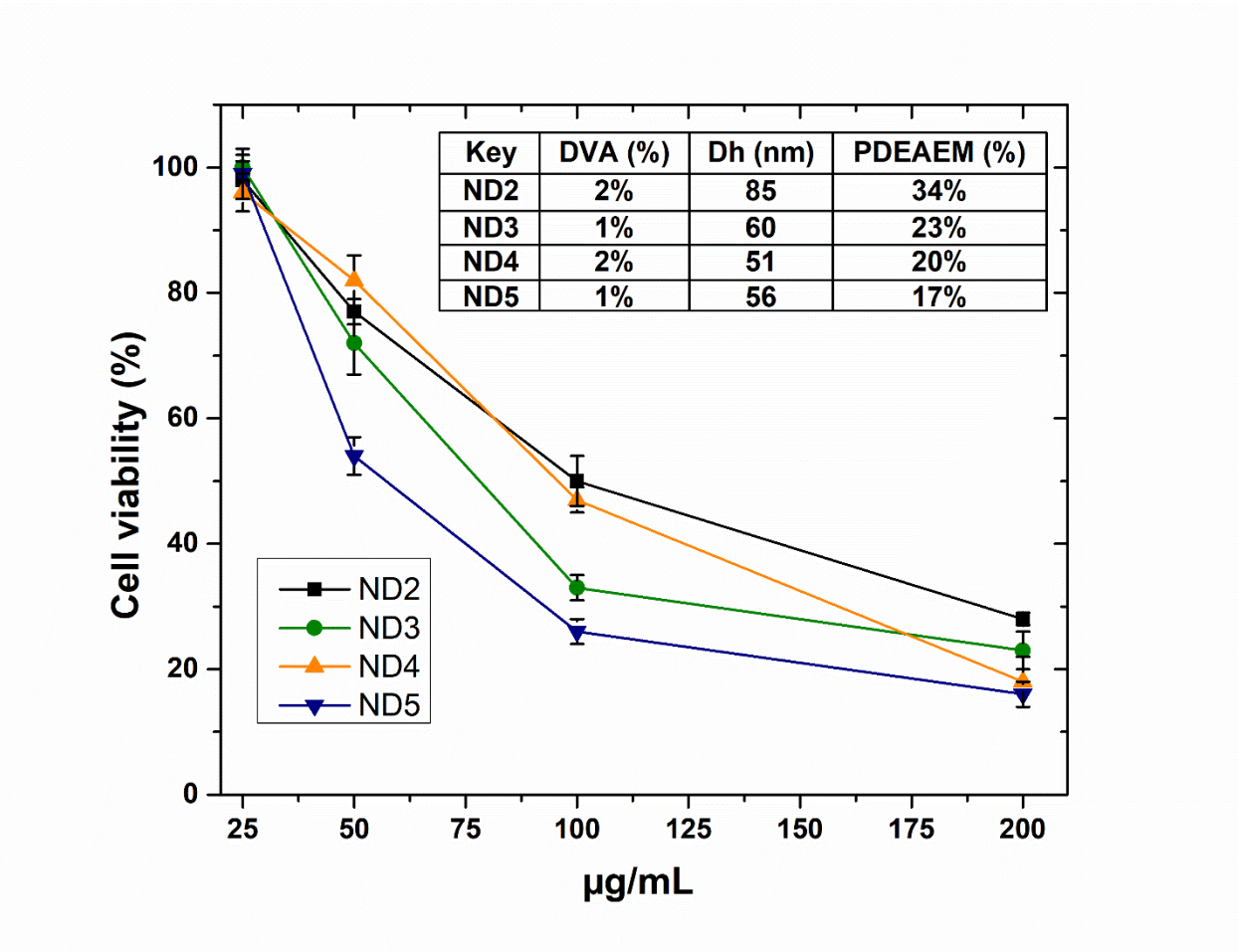

Figure S24. Cytotoxic effect of empty degradable PDEAEM-*core*-PEG-*shell* nanogels (using DVA crosslinker).The cell viability (%) of cells is expressed as function of untreated cells (C-). The results represent the average ± SEM of triplicates. Positive control (C+) 5 % DMSO. $p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$ vs C- (unpaired *t*-Student's test).

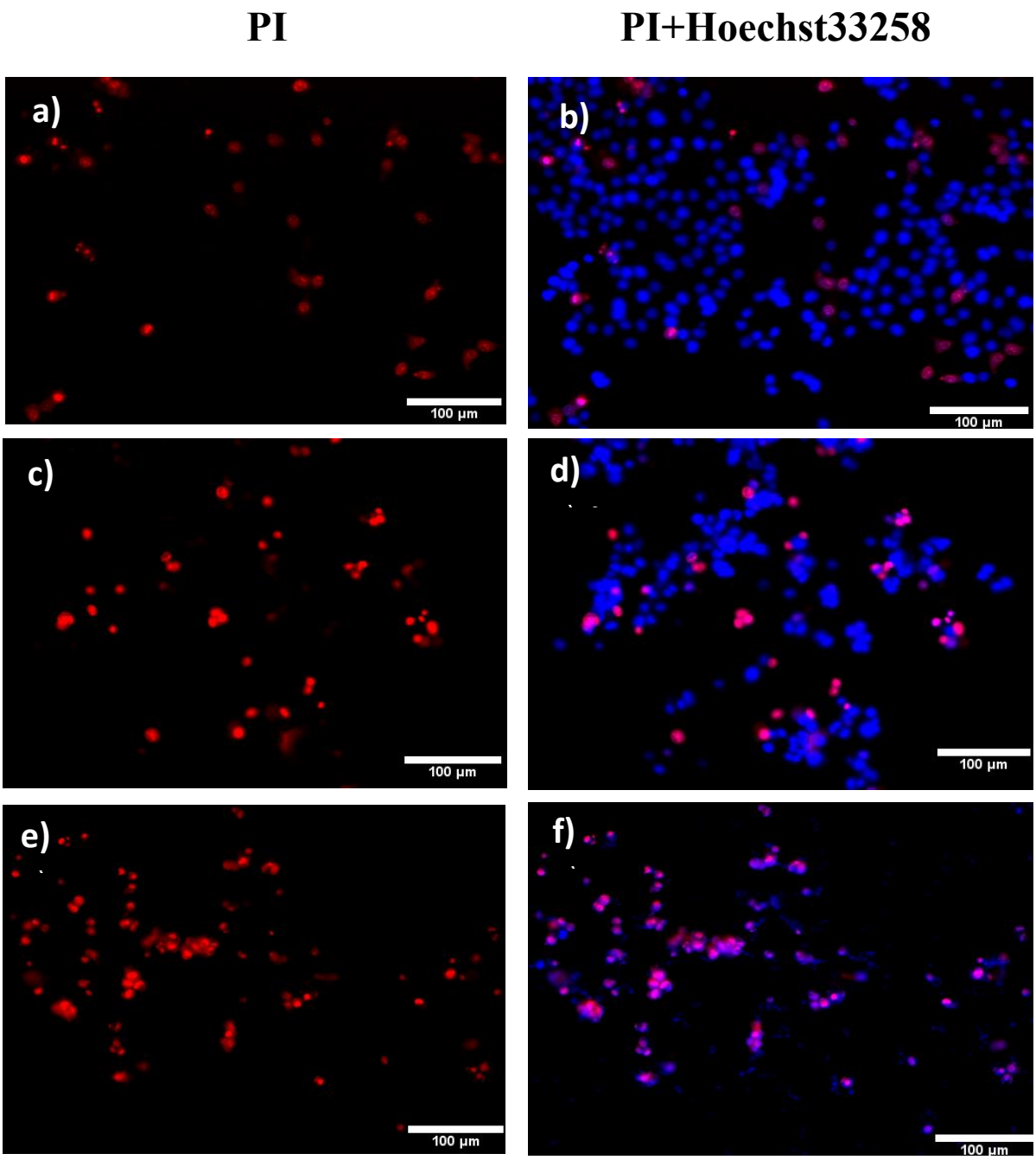

Figure S25. Fluorescence microscope images of human colon cancer cell line (HCT-116): cells incubated with free CUR (5µg/mL) for: a)b)6 h, c)d)12 h and e)f)24 h. Representative images showing cells treated with propidium iodide (PI) that is used to identify necrotic or apoptotic cells (red, left hand side) and cell nuclei were counterstained with Hoechst 33258 in blue (right hand side superimposed images).

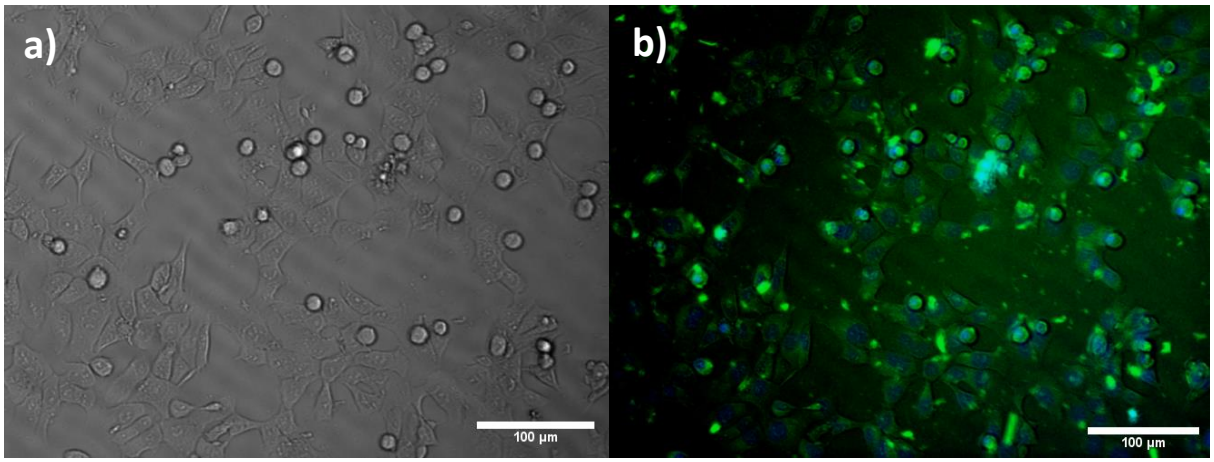

Figure S26. Fluorescence microscope images of human colon cancer cell line (HCT-116) treated with Hoechst 33258: a) CUR loaded non degradable nanogels (NE2, EGDMA crosslinked, CUR:1µg/mL, white light), b) CUR loaded non degradable nanogels (NE2, EGDMA crosslinked, CUR:1 µg/mL, blue and green light superimposed images).

Table S1. Acute toxicity studies for LD_{50} determination by intraperitoneal route of NB4 nanogel compound in female mice CD1.

Bodyweigth (g)

Dose	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
10 mg/kg	23.8	24.7	25.6	25.2	25.4	25.2	26.5	26.8	27.3	27.4
20 mg/kg	21.8	22.5	22.7	23.0	22.8	22.8	23.0	23.7	24.7	25.1
40 mg/kg (1)	19.0	19.0	20.2	19.9	19.6	20.7	21.4	22.0	22.4	22.8
40 mg/kg (2)	24.9	25.6	25.2	24.8	23.5	26.3	27.3	27.3	27.3	27.2
40 mg/kg (3)	23.7	23.5	23.7	23.8	23.8	24.2	24.7	25.2	25.9	27.2

Table S2. Acute toxicity studies for LD_{50} determination by intraperitoneal route of NE2 nanogel compound in female mice CD1.

Bodyweigth (g)

Dose	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10
10 mg/kg	24.2	25.3	25.9	26.2	26.1	27.6	28.4	28.6	29.9	29.5
20 mg/kg	21.3	21.6	22.3	23.0	23.5	23.4	24.4	24.9	25.3	25.4
40 mg/kg (1)	23.5	21.6	22.8	23.0	23.3	23.9	24.4	25.1	25.3	25.4
40 mg/kg (2)	25.1	23.3	23.8	23.4	25.1	25.7	26.5	26.6	26.7	26.5
40 mg/kg (3)	21.5	19.9	20.3	21.4	21.2	21.8	22.5	23.1	24.1	26.5