Supporting Information for:

Reactive Self-Assembly of Polymers and Proteins to Reversibly Silence a Killer Protein

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Determination of the number of accesible and reactive cysteine (thiols) on caspase-3. A 1.5 mL stock of 10 μM caspase-3 was purified using a NAP25 (GE Healthcare) size exclusion column to fully exchange the buffer to 20 mM Tris pH 8.0 and eliminate all DTT. The caspase-3 (3.6 μM) was then incubated at room temperature with 50 μM of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) for 30 minutes. The absorbance of this reaction was then recorded at 412 nm. The Beer-Lambert law was then used on the corrected absorbance with a molar absorptivity for DTNB of 14,150 $M^{-1}cm^{-1}$ and a pathlength of 1 cm. This yielded 5 DTNB molecules per monomer of caspase, indicating that there are 5 accessible and reactive cysteine thiols per monomer of caspase-3.

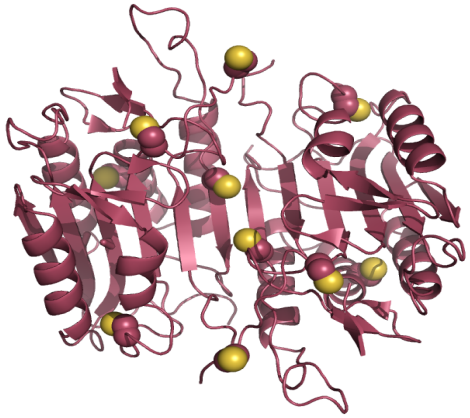

Figure S1. Surface exposed cysteine residues in caspase-3

Labeling of Caspase-3 with FITC. Fluorescein isothiocyanate isomer I (FITC) was dissolved in a 100 mM sodium bicarbonate solution pH 9.0 to a concentration of 1 mg/mL. Caspase-3 (2.65 mg) was diluted to 2 mL in 100 mM sodium bicarbonate buffer pH 9.0 with a total of 1.5 mg of FITC present. This reaction was protected from light and allowed to stir overnight at 4°C. The resulting FITC-labeled caspase-3 was dialyzed in 50 mM Tris pH 7.5, 50 mM NaCl, and 2 mM DTT to remove excess FITC. The caspase was then concentrated using a 3,000 Da spin filter and the concentration was measured by UV-vis absorption spectroscopy.

NMR Spectrum of p(PEGMA-co-PDSMA) (P1):

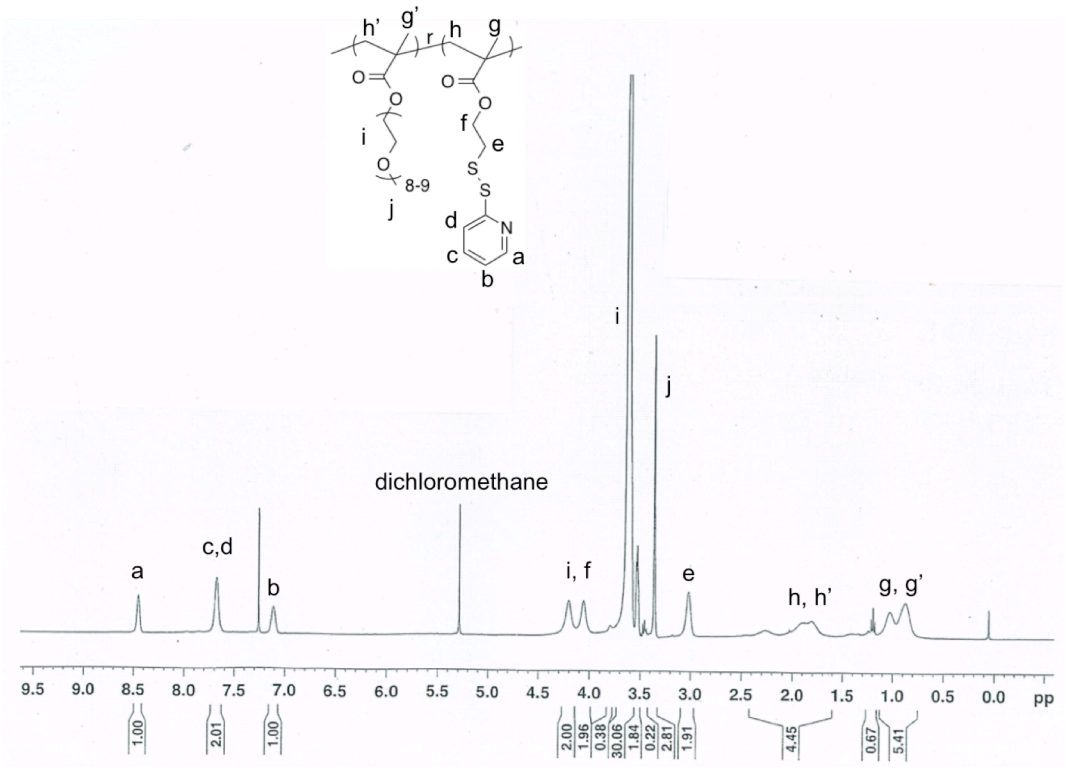

Figure S2. NMR spectra of p(PEGMA-*co*-PDSMA)

Synthesis of CRRR peptide. This peptide was prepared following a previously reported procedure (*J. Am. Chem. Soc.*, **2012**, *134*, 6964-6967) except utilizing a different resin. Rink Amide AM resin (200-400 mesh) was selected as the solid support to prepare the peptide using the solid phase synthesis. Peptide was used without further purification. ^{1}H NMR (400 MHz, D_2O) δ: 4.45-4.52, 4.30-4.41, 4.03-4.11, 3.15-3.26, 2.92-3.01, 1.73-1.96, 1.55-1.96. MS (FAB): exact mass calculated: 590.7. Found: 590.0

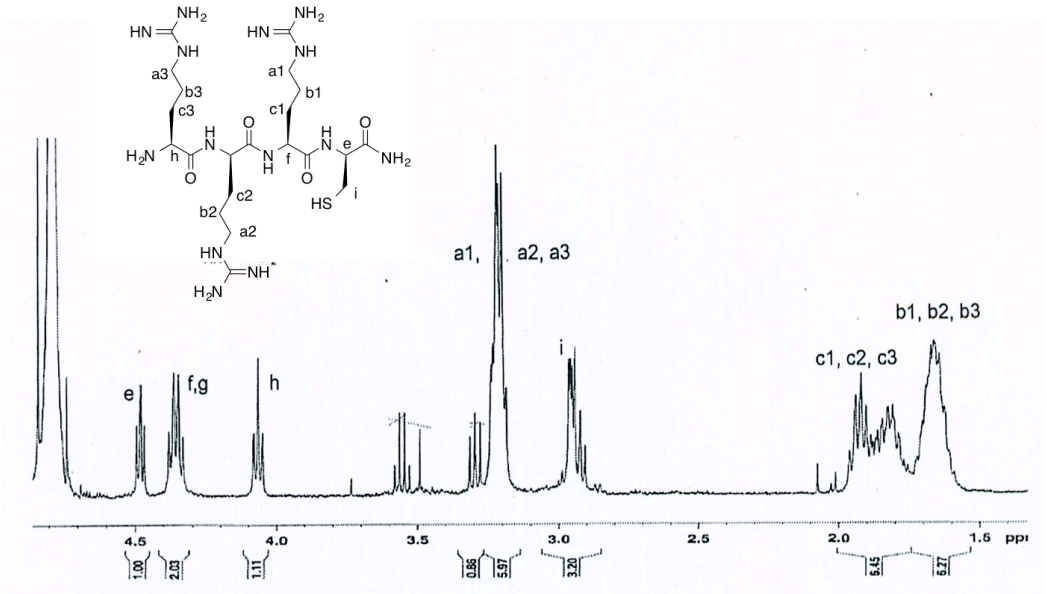

Figure S3. NMR spectrum of the CRRR peptide

Crosslinking density, caspase conjugation and peptide functionalization. The crosslinking density was determined following the reported procedure (*J. Am. Chem. Soc.*, **2012**, *134*, 6964-6967) by calculating the amount of the byproduct, 2-pyridinethione, and its known molar extinction coefficient (8.08 x 10^3 M^{-1} cm^{-1} at 343 nm) (*Bioconjugate Chem.* **2006**, *17*, 1376-1384). The functionalization of the nanogels with CRRR peptide was evaluated by the further formation of 2-pyridinethione (increase in the absorption spectra at 343 nm).

Example 1: Calculation of crosslinking density in NG-FITC-Casp-In.

PEG:PDS = 48:52

Molecular weight of the polymer repeat unit: 360.6 g/mol

From the nanogel synthesis reaction mixture, a solution of 0.1 mg/mL = 0.05 mg/0.5mL was prepared (total volume of 0.5 mL).

1) Calculate mol of PDS in the solution:

[0.05 mg / (360.6 g/mol)] * (0.52) = 7.2 x 10^{-8} mol PDS

2) Calculate mol of 2-pyridinethione:

By Beer-Lambert law, A = εbc, and the absorbance at 343 nm of the solution: 0.8274

Therefore, c = 0.8274 / [(8.08 x 10^{3} M^{-1} cm^{-1}) (1 cm)] = 1.02 x 10^{-4} M

Since a total volume of 0.5 mL was used, the final mol of 2-pyridinethione is

= 5.1 x 10^{-8} mol

3) It is 70.8 mol% of total PDS unit (7. 2 x 10^{-8} mol). We assume that two 2-pyridinethione are from one disulfide formation and PDS unit is 52 mol% of total polymer:

(70.8 % / 2) * 0.52 = 18.5 % crosslinking density.

Example 2: Calculation of crosslinking density in NG-FITC-Casp-In^{RRR}.

From the nanogel synthesis reaction mixture, a solution of 0.05 mg/mL was prepared (total volume of 1 mL).

1) Calculate mol of PDS in the solution:

[0.05 mg / (360.6 g/mol)] * (0.52) = 7.2 x 10^{-8} mol PDS

2) Calculate mol of 2-pyridinethione:

By Beer-Lambert law, $A = \varepsilon bc$, and the absorbance at 343 nm of the solution: 4.01×10^{-1}

Therefore, $c = 4.01 \times 10^{-1} / [(8.08 \times 10^{3}\ M^{-1}\ cm^{-1})\ (1\ cm)] = 4.96 \times 10^{-5}$ M

Since a total volume of 1 mL was used, the final mol of 2-pyridinethione is

$= 4.96 \times 10^{-8}$ mol

3) It is 68.9 mol% of total PDS unit ($7.\ 2 \times 10^{-8}$ mol). We assume that two 2-pyridinethione are from one disulfide formation and PDS unit is 52 mol% of total polymer:

(68.9 % / 2) * 0.52 = 17.9 % crosslinking density.

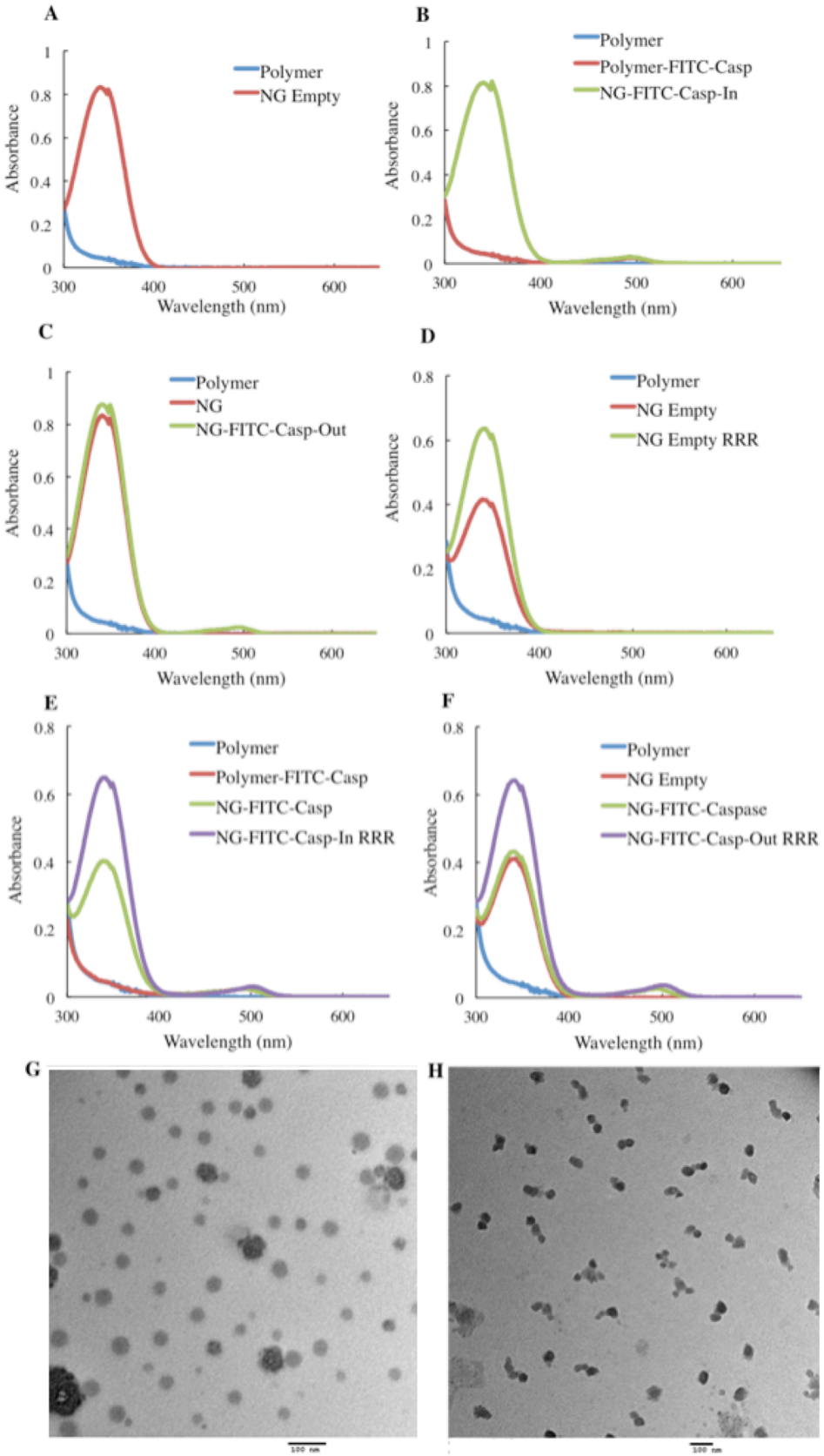

Figure S4. Absorption spectra of nanogel-caspase conjugates synthesis. A) NG Empty (0.1 mg/mL) B) NG-FITC-Casp-In (0.1 mg/mL) C) NG-FITC-Casp-Out (0.1 mg/mL) D) NG Empty^{RRR} (0.05 mg/mL) E) NG-FITC-Casp-In^{RRR} (0.05 mg/mL) F) NG-FITC-Casp-Out^{RRR} (0.05 mg/mL). TEM images of G) NG-Casp-In H) NG-Casp-Out (scale bar is 100 nm).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) studies. Samples of NG-Empty (50 μg), NG-Casp-In (50 μg), NG-Casp-Out (50 μg), NG-Empty^{RRR} (50 μg), NG-Casp-In^{RRR} (50 μg) and NG-Casp-Out^{RRR} (50 μg) were run on a 16% acrylamide gel under non-reducing conditions (no DTT present). Identical samples were then treated with 100 mM DTT and allowed to sit at 25 °C for one hour before running SDS-PAGE. Using a standard curve of known caspase concentrations the caspase released from the nanogels was then quantified using the Bio-Rad Image Lab™ software. This yielded the precise amount of caspase released per 50 μg of nanogel.

Enzymatic degradation experiment. Sample solutions of nanogels and nanogel-caspase conjugates (1 mg/mL) with a final volume of 0.050 mL were prepared. The concentration of caspase-3 in the conjugate solutions was estimated to be 14 μM based on the feed amount during its synthesis (caspase-3 molecular weight = 28,500). The protein was denatured by adding acetonitrile (20% of the total volume) and the solutions were left at room temperature for 10 minutes. The digestion of caspase-3 was performed by adding trypsin from porcine pancreas, 4:1 ratio (caspase-3:trypsin), and incubated for 20 hours at room temperature. Nanogel solution was exposed to the same amounts and conditions as those for nanogel-caspase conjugates. The matrix solution was prepared by mixing 22.5 mg of α-cyano-hydroxycinnamic acid in 350 μL tetrahydrofuran, 150 μL water and 6 μL of trifluoroacetic acid. 10 μL of each of the sample solutions and 10 μL of the matrix solution were mixed and spotted on a MALDI target.

Caspase activity after lyophilization of samples:

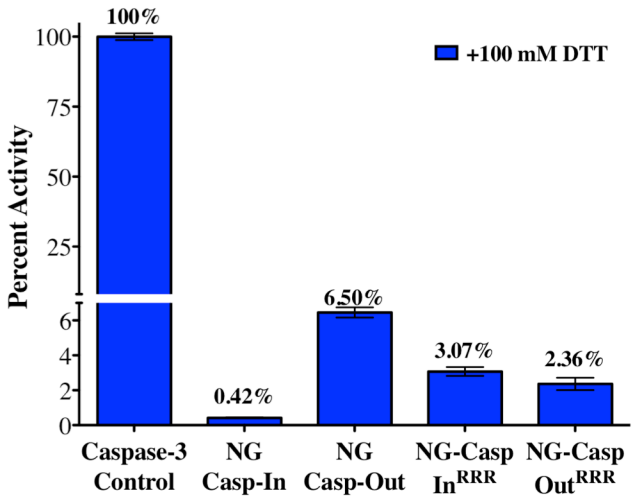

Figure S5. Caspase-3 activity recovered after an early protocol for the construction of caspase-containing nanogels involving lyophilization. 50 nM caspase-3 control and 50 nM released from nanogels.

Cell Internalization Studies. Cell internalization studies were performed using Zeiss 510 META confocal laser scanning microscopy. HeLa cells were cultured in T75 cell culture flask containing Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) with 10% fetal bovine serum (FBS) supplement. The cells were seeded at 100,000 cells/mL in cover slip-bottomed Petri dishes and allowed to grow for 1 day at 37 °C in a 5% CO_2 incubator. The cells in 1 mL of culture medium were treated with nanogels and nanogel-FITC-caspase conjugates (0.5 mg/mL) and were incubated for 4 hours at 37 °C before monitoring the cells by confocal microscopy. The nucleus of the cells was stained by the addition of DRAQ5 (5 μM). All images were taken using 63x oil immersion objectives (excitation at 488 nm for FITC and 543 nm for DRAQ-5).

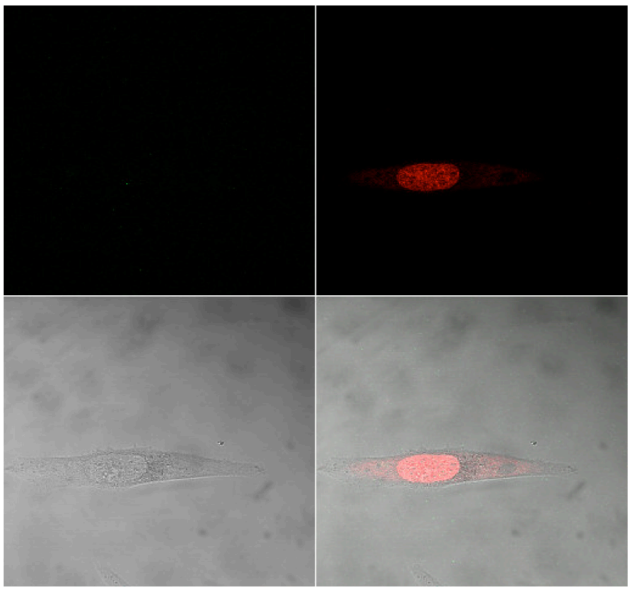

Figure S6. No cellular internalization after incubating the cells for 4 hours with FITC-caspase-3. Within each image set, top left is the FITC channel, which show green color for caspase-3 and top right is the DRAQ5 channel, which shows red color for the nucleus. Bottom left is the DIC image and bottom right is the overlap of all three.

Apoptosis studies. HeLa cells were cultured in T75 cell culture flasks using DMEM/F12 with 10% FBS supplement. The cells were seeded at 10,000 cells/well/200 μL in a 96 well tissue culture plate and allowed to grow for 24 hours under incubation at 37 °C in 5% CO_2. The cells were then treated with different concentrations of nanogel-caspase conjugates and were incubated for another 24 hours. Cell viability was measured using Alamar Blue assay with each data point measured in triplicate. Fluorescence measurements were made using the plate SpectraMax M5 by setting the excitation wavelength at 560 nm and monitoring emission at 590 nm on a black 96 well flat bottom plate.

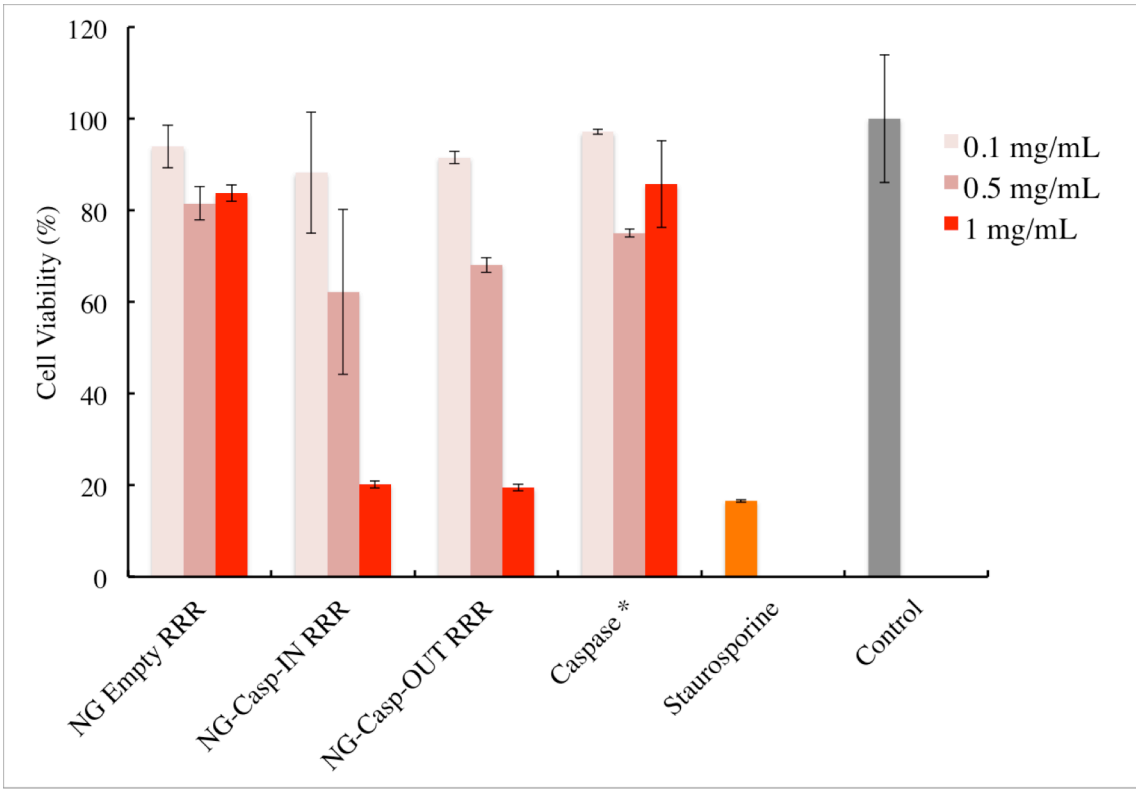

Figure S7. Apoptosis experiment performed with nanogel-caspase^{RRR} on day two.

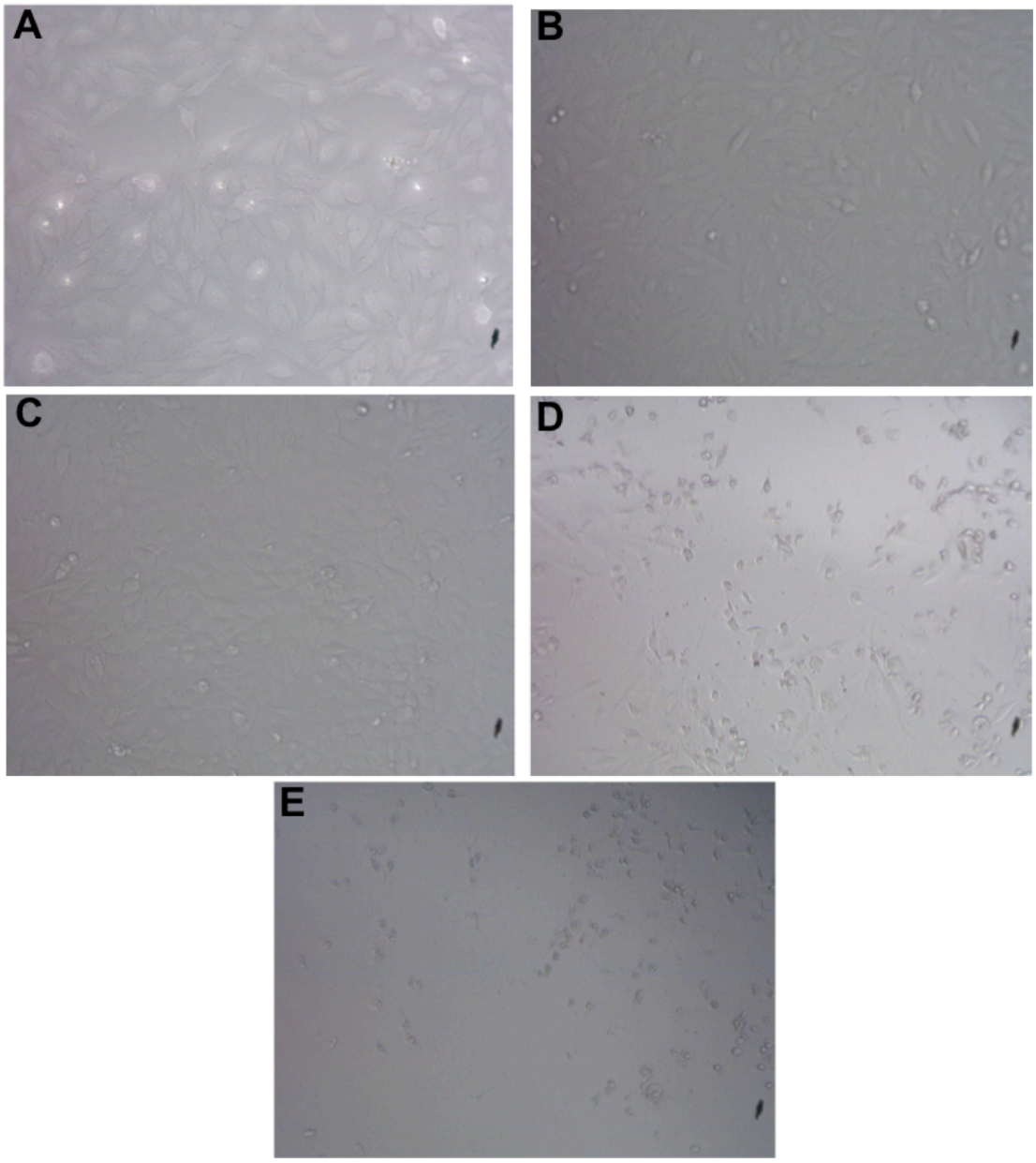

Figure S8. HeLa cells after incubation for 24 hours with nanogel-caspase conjugates. A) Untreated cells B) 1 mg/mL NG Empty C) 1mg/mL NG-Casp-In D) 1 mg/mL NG-Casp-Out E) 1 μM staurosporine