Electronic Supplementary Material (ESI) for Nanoscale.

Supplementary Information

ND pretreatment

NDs were supplied by Microdiamant Switzerland (MSY 0-0.05). The NDs were oxidized by air in a furnace (Thermolyne 21100 tube) at 510 °C for 5 h. Subsequently, the NDs were treated with a mixture of H_2SO_4 and HNO_3 (9:1) at 90 °C for 3 days and washed with water, 1 M NaOH and 1 M HCl. They were washed an additional 5 times with water and then freeze-dried. Purified ND powder (120 mg), containing approximately 100–200 ppm of natural nitrogen impurities, was irradiated in 5% aqueous colloidal solution for 210 min with a 15.7 MeV proton beam (4.9×10^{17} protons/ml) extracted from the isochronous cyclotron U-120M. The irradiated material was annealed at 900 °C for 1 h and subsequently oxidized for 4 h at 510 °C. The NDs were again treated with a mixture of H_2SO_4 and HNO_3 (9:1) at 90 °C for 3 days. The resulting material was washed with water, 1 M NaOH and 1 M HCl and an additional 5 times with water. The particles were dissolved in water (2 mg/ml) and filtered using a 0.2 μm PVDF filter, yielding ND-COOH colloid.

Coating of NDs with a methacrylate-terminated thin silica layer

Polyvinylpyrrolidone (M = 10,000, 96 mg, 9.6 μmol, purchased from Sigma-Aldrich) was dissolved in water (204 ml) and sonicated for 10 min in an ultrasonic bath. ND-COOH colloid (6 ml, 2 mg/ml) was added, and the mixture was stirred for 24 h. The colloid was then concentrated by a two-step centrifugation. In the first step (40,000 rcf, 1 hour), the volume was reduced to approximately 12 ml. The second centrifugation step (30,000 rcf, 30 min) was performed in microvials, and the solvent volume was reduced to approximately 0.2 ml. Sedimented NDs were resuspended in ethanol (12 ml) in a round bottom flask and sonicated in an ultrasonic bath for 2-4 min. Tetraethyl orthosilicate (84.06 mg, 405 μmol, purchased from Sigma-Aldrich) and 3-(trimethoxysilyl)propylmethacrylate (31.35 mg, 126 μmol, purchased from Sigma-Aldrich) were added to the round bottom flask. Ammonia (25%, 498 μl) was added after 20 s sonication in an ultrasonic bath. The reaction mixture was stirred for 14 h. The product was purified by centrifugation (14,000 - 25,000 rcf, 15 min) with ethanol (12 ml, 2 times).

Coating of NDs with a polymer layer

HPMA [700 mg, 4.82 mmol, synthesized according to published procedures[1] and freshly recrystallized twice from a mixture of acetone-hexane (1:3 v/v)] and N-propargyl acrylamide (35 mg, 0.06 mmol, synthesized by acryloylation of propargylamine with acryloyl chloride[2]) were dissolved in DMSO (2.1 ml). 2,2′-Azobis(2-methylpropionitrile) (AIBN, 200 mg, 1.22 mmol, recrystallized by thickening an ethanol solution on a rotary evaporator at a maximum temperature of 30 °C) was added to the mixture. The mixture was filtered using a 0.2 μm polytetrafluorethylene microfilter. Methacrylate-terminated ND particles (2 mg) were added. The reaction proceeded for 3 days under argon at 55 °C. The particles were centrifuged (21,000 rcf, 30 min) and purified by centrifugation with ethanol (25,000 rcf, 30 min, 1 ml, 3 times) and water (30,000 rcf, 30 min, 1 ml, 5 times).

FND colloidal stability studies

The coating method with HPMA polymer was improved compared to our recently published method[3] to yield **FNDs** with even better colloidal stability and fewer non-specific protein interactions. FNDs synthesized according to the previously published method were stable in biological solutions such as media or buffers but immediately began to aggregate in 0.5 M (and higher) NaCl concentrations, according to dynamic light scattering (DLS, Fig. 1S). The newly prepared **FNDs** are stable even after 24 h incubation in 1 M NaCl (Fig. 2S). DLS was recorded with a Zetasizer Nano ZS system (Malvern Instruments) at 25 °C. Sample concentrations were 0.1 mg/ml.

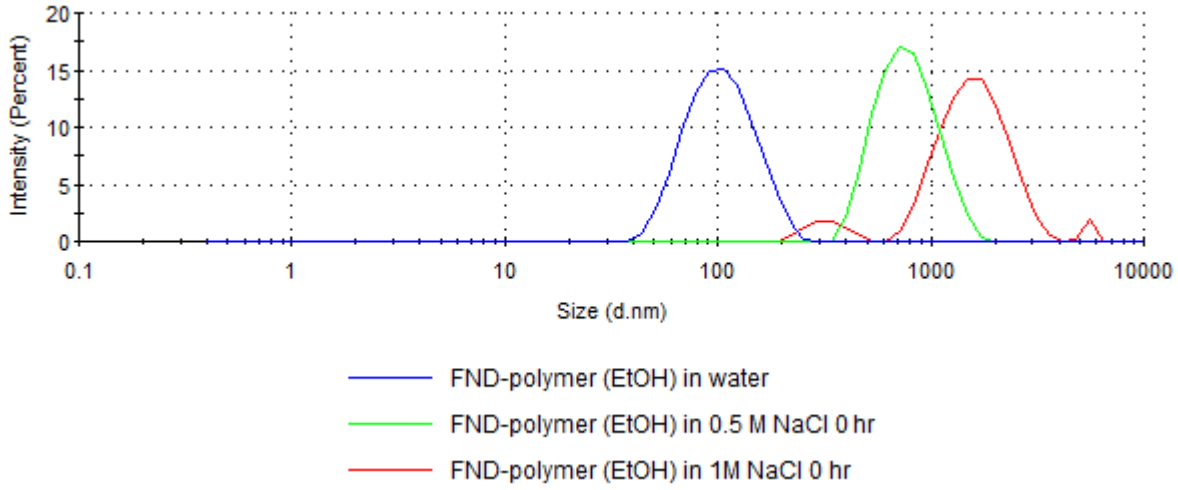

Fig. 1S Dynamic light scattering (DLS) of the size distribution of **FNDs** synthesized according to our previously published method[3] in solutions with various concentrations of NaCl. The concentration of the colloids was 0.1 mg/ml.

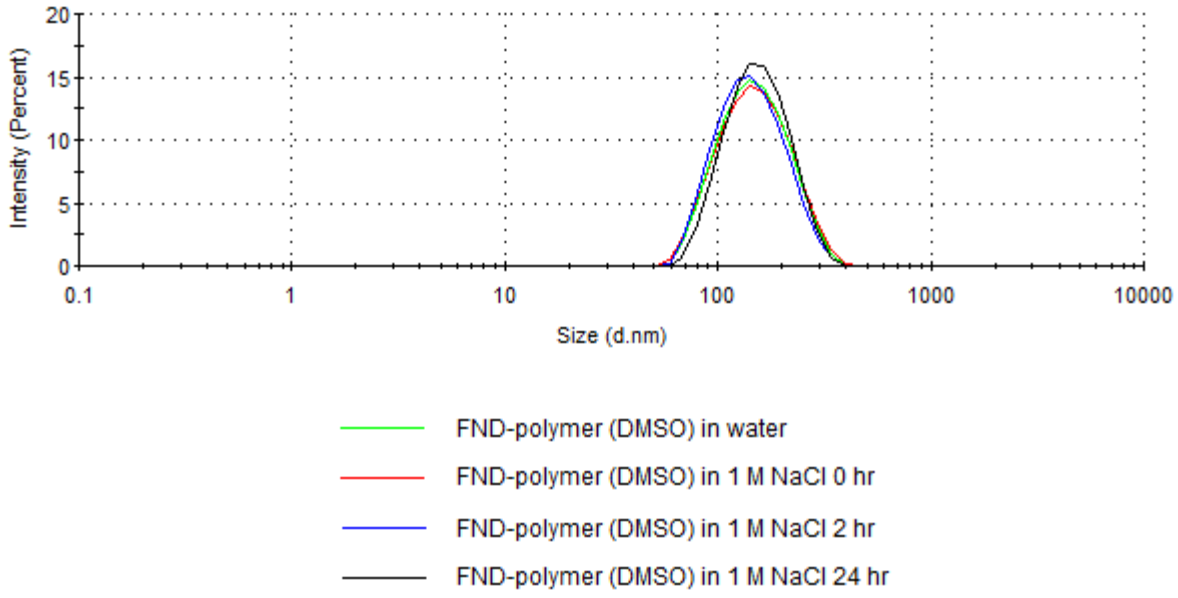

Fig. 2S Dynamic light scattering (DLS) of the size distribution of newly prepared **FNDs** in solutions containing various NaCl concentrations. The concentration of colloids was 0.1 mg/ml.

Functionalization of coated FNDs by click reaction

FNDs were modified with Alexa Fluor 488-azide (purchased from Life Technologies) and cRGD-azide (cyclo(Arg-Gly-Asp-D-Phe-Lys), purchased from Peptides International). FNDs were modified with these two ligands in consecutive reactions utilizing the same surface functionalities. Washing procedures were employed after both modification steps.

Stock solutions for copper(I)-catalyzed azide-alkyne cycloaddition reactions were prepared in water, except for the Alexa Fluor 488 stock, which was prepared in DMSO. The solutions of $CuSO_4 \cdot 5H_2O$ and tris(3-hydroxypropyltriazolylmethyl)amine (THPTA, synthesized according to published procedures[4]) were premixed (in a 1:2 concentration ratio) before they were added to the reaction mixture to yield final concentrations of 0.32 mM and 0.64 mM, respectively. The mixtures were filled to the final volume with water.

For the reaction of modified FNDs with Alexa Fluor 488-azide, the reactants were mixed in the following order and final concentrations: colloid of poly(HPMA)-alkyne modified FNDs (0.4 mg in a final reaction volume of 512 µl), Alexa Fluor 488-azide (20 µM), Cu-catalyst solution (see above), aminoguanidine hydrochloride (5 mM) and a freshly prepared solution of sodium ascorbate (5 mM). The reaction mixture was well-sealed, mixed and left for 3 h with no stirring. Modified **FNDs** were isolated by centrifugation (26,000 rcf, 10 min) and twice washed with 1 ml water. Half (0.2 mg) of the Alexa Fluor 488-modified **FNDs** was reacted in a click reaction with cRGD-azide (160 µM) under the same conditions. Polymer-coated **FNDs** modified with Alexa Fluor 488 (**FNDs**) and polymer-coated **FNDs** modified with Alexa Fluor 488 and cRGD (**FND-cRGD**) were both treated the same and were washed with water (1 ml, 7 times).

Cell culture

Human glioblastoma-astrocytoma U-87 MG cells (ATCC® HTB-14™) were cultivated in Eagle's Minimum Essential Medium (EMEM, LGC Standards Sp.z.o.o., cat. no. ATCC-30-2003) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 1% (v/v) non-essential amino acids (Sigma-Aldrich, cat. no. M7145) and antibiotics (100 I.U./ml penicillin, 100 µg/ml streptomycin; Sigma-Aldrich) at 37 °C in a humidified atmosphere containing 5% CO_2. Sub-confluent cells were subcultured every 3-4 days using a solution of 0.25% (w/v) trypsin and 0.53 mM EDTA (Sigma-Aldrich).

Cell viability assay

The CellTiter-Glo® Luminiscent Cell Viability Assay (Promega) was used to test U-87 MG cell viability.

U-87 MG cells were cultivated as described above. When they reached the exponential growth phase, cells were harvested and seeded into a 96-well white microtiter plate (NUNC) at 4,000 cells per well. Each well contained 90 µl cell suspension. About 24 h prior to the experiment, the growth media was replaced with 90 µl fresh media. The next day, 10 µl of 10-times concentrated modified **FND** particles were applied in pentaplicates to wells (final concentration 50 µg/ml). Free cRGD and the known apoptosis inducer staurosporine (Sigma-Aldrich) were added to the cells at concentrations of 100 µg/ml and 0.3125–5 µM, respectively. Into wells containing controls (cells without test compounds) and blanks (pure medium without cells), a volume of water equal to the volume of the compound solutions added to the experimental wells was added. After 1 h incubation with **FND** particles or compounds, the media in each well was replaced with fresh media without the tested substances and left for an additional 2 h. Then, the viability assay was performed as recommended by the manufacturer. Briefly, the loaded 96-well plate and CellTiter-Glo® substrate were equilibrated to room temperature prior to analysis (30 min). Then, the reconstituted reagent (100 µl) was added to each well, and the 96-well plate was mixed for 2 min at 370 RPM on an orbital shaker in the dark. Subsequently, the luminescent signal was allowed to stabilize for 15 min at room temperature. Luminescence was recorded using a microplate luminometer reader (Tecan GENios, Tecan, Austria). Blanks were subtracted from experimental and control measurements. The percentage of viable U-87 MG cells was counted as the ratio of luminescence from wells containing U-87 MG cells and test compounds to the luminescence signal from an untreated population control. This ratio represents the mean ± SD from 1 independent measurement performed in pentaplicate wells (or 1 independent measurement performed in triplicate wells for staurosporine). The apoptosis inducer staurosporin showed toxicity in a concentration-dependent manner (Fig 3S). The change in cell morphology after adding 2.5 µM staurosporine is shown in Fig. 4S (from inverted microscope Zeiss Axio Observer.A1).

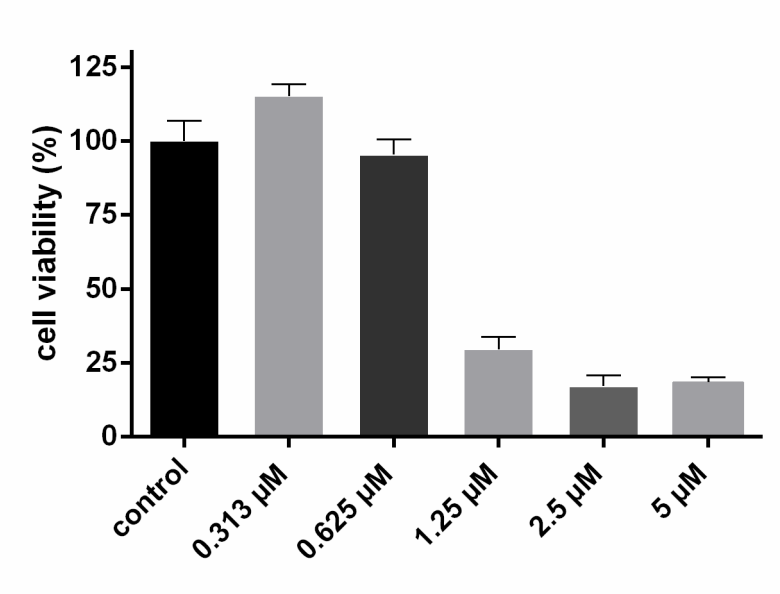

Fig. 3S Cell viability assay based on ATP quantification in cell lysates after adding the apoptosis inducer staurosporine in different concentrations. The luminescence intensity correlates with the ATP level and thus with the quantity of metabolically active (viable) cells. Experiments with 0.313 µM and 0.625 µM staurosporin are statistically not distinguishable from the control, whereas experiments with 1.25 µM, 2.5 µM an 5 µM staurosporin concentration are statistically different from control (ANOVA, $\alpha = 0.05$).

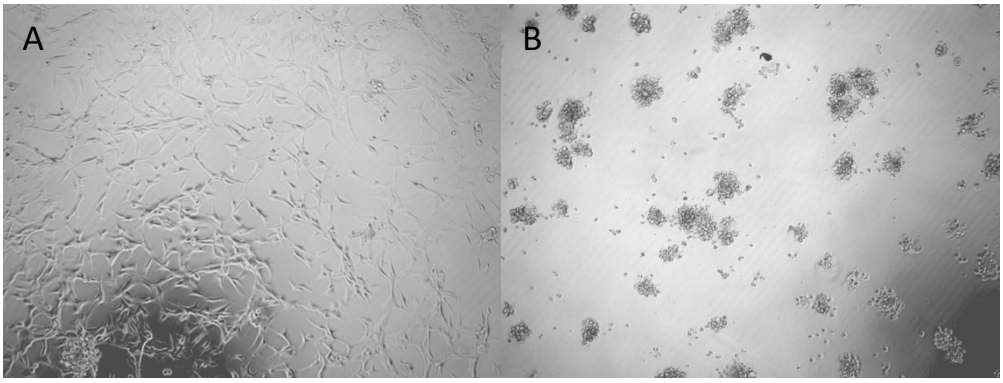

Fig. 4S Microscope observation of U-87 MG cells before (A) and after (B) adding 2.5 µM staurosporine, an inducer of apoptosis.

Flow cytometry

Five days prior to experimental treatment, U-87 MG cells were harvested in the exponential growth phase and seeded into a 24-well plate at 40,000 cells per well. Each well contained 1 ml

cell suspension. Within the cultivation period, the growth medium in the wells was replaced once with fresh medium. On the day of treatment, the cells reached approximately 60-70% confluence. In each experiment, the following 1 h treatments were performed in duplicate: control (growth medium + the corresponding amount of water), **FND** (final concentration of 50 µg/ml), **FND-cRGD** (final concentration of 50 µg/ml). Additionally, pretreatment of cells with 100 µg/ml cRGD for 30 min was performed before **FND** and **FND-cRGD** were applied to the wells (50 µg/ml). Subsequent 60 min incubation was carried out in the dark at 37 °C in a humidified atmosphere containing 5% CO_2. After treatment, cells were harvested by scraping, washed twice with 900 µl cold PBS, resuspended in 200 µl PBS, filtered through a 35-µm nylon mesh and analyzed with a BD LSRFortessa™ cell analyzer (Becton Dickinson, San Jose, CA). Ten thousand events were acquired for each analysis. Fluorescence was excited at 488 nm and detected in spectral range 515–545 nm. BD FACSDiva Software 6.0 was used to generate histograms (see Figure 5S) and analyze data. The results represent the mean ± SD from 2 independent measurements performed in duplicate and triplicate wells.

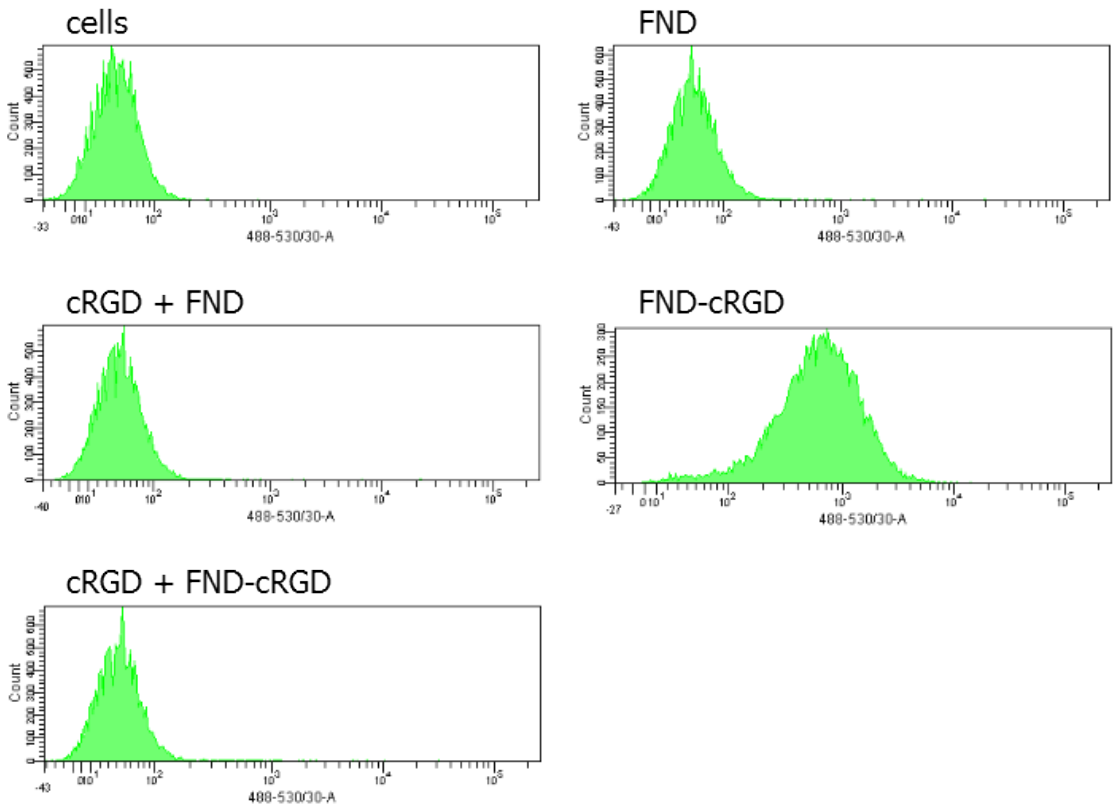

Fig. 5S Histograms obtained from flow cytometry measurements.

Confocal microscopy

Five days prior to experimental treatment, U-87 MG cells were harvested in the exponential growth phase and seeded into Petri dishes (35 mm glass bottom dish with 4 chambers, BioPort Europe, s.r.o., cat. no. D35C4-20-1.5-N) at 40,000 cells per well. Each well contained 1 ml cell suspension. Within this cultivation period, the growth medium in wells was replaced once with fresh medium. On the day of treatment, the cells reached approximately 60-70% confluence. In each experiment, the following 1 h treatments were performed: control (growth medium + the corresponding amount of water), **FND** (final concentration of 50 μg/ml), **FND-cRGD** (final concentration of 50 μg/ml). Additionally, pretreatment of cells with 100 μg/ml cRGD for 30 min was performed before **FND** and **FND-cRGD** were applied to the wells (50 μg/ml). Subsequent 60 min incubation was carried out in the dark at 37 °C in a humidified atmosphere containing 5% CO_2. After treatment, cells were washed twice with 900 μl cold PBS and fixed with a 2% formaldehyde solution in PBS for 10 min. After fixation, cells were washed twice with 900 μl PBS and stored in 1 ml PBS.

A confocal imaging of internalized **FNDs** was performed on Carl Zeiss LSM 780 confocal microscope, equipped with two solid state lasers (405 nm, 561 nm) lasers, in-tune laser (tunable laser), oil-immersion objective (Plan-Apochromat 63x/1.40 Oil DIC M27) and ultrasensitive spectral 32+2 channel detector.

Firstly, the measurement of Alexa Fluor 488 dye bound to **FNDs** was done with following setup: excitation at 488 nm by argon ion laser with 3.5 μW output power (at the sample plane), 90 μm pinhole, pixel dwell time 153 μs, pixel size 81 nm, spectral detector in the 493-630 nm range (master gain set to 850 V, digital gain 1). Bright-field images were taken by using transmitted light (PMT detector at 375 V, digital gain 1) concomitantly. An extensive bleaching for reducing unwanted background was performed after that; 30 cycles with 153 μs pixel dwell time using combined power of lasers at excitation wavelengths of 405 nm (~ 1.18 mW), 488 nm (~ 0.62 mW), 514 nm (~ 0.26 mW), 532 nm (~ 0.4 mW) and 561 nm (~ 1.69 mW).

Finally, intrinsical fluorescence of **FNDs** was examined. **FNDs** were excited using the solid state 561 nm laser (~1,69 mW), scanned with pixel size 81 nm, pixel dwell time 153 μs with 4× line averaging, 90 μm pinhole and emitted light was collected by spectral detector setup in the 569-691 nm range, in photon counting lambda mode (14 channels, digital gain 1). The measured

emission spectrum of **FNDs** in cells has maximum at 663 nm, therefore signal between wavelengths 659 - 667 nm was used for visualization of **FNDs** in cells and signal in range 624-691 nm was used for subsequent quantitative colocalization image analysis.

The fluorescence images were collected with ZEN 2011 software and processed in the GNU Image Manipulation Program or in the ImageJ software[5] with JACoP colocalization plug-in. Because of different intensity in the Alexa Fluor 488 fluorescence channel and FND channel, intensity of Alexa Fluor 488 channel was multiplied by factor 0.6 before merging the figures. Quantitative colocalization analysis was performed by JaCoP plugin. Briefly after background subtraction, threshold levels were automatically computed by Coste's method and correlation coefficients were computed.[6]

Statistical analysis

The ANOVA analysis was done using Data Analysis tool in Microsoft Excel 2010.

References

1. S. K. Filippov, P. Chytil, P. V. Konarev, M. Dyakonova, C. Papadakis, A. Zhigunov, J. Plestil, P. Stepanek, T. Etrych, K. Ulbrich, and D. I. Svergun, *Biomacromolecules*, 2012, **13**, 2594–2604.
2. H. Macková, V. Proks, D. Horák, J. Kučka, and M. Trchová, *J. Polym. Sci. Part Polym. Chem.*, 2011, **49**, 4820–4829.
3. I. Rehor, H. Mackova, S. K. Filippov, J. Kucka, V. Proks, J. Slegerova, S. Turner, G. Van Tendeloo, M. Ledvina, M. Hruby, and P. Cigler, *ChemPlusChem*, 2014, **79**, 21–24.
4. V. Hong, S. I. Presolski, C. Ma, and M. G. Finn, *Angew. Chem. Int. Ed.*, 2009, **48**, 9879–9883.
5. Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2014.
6. S. Bolte & F. P. Cordelieres, A guided tour into subcellular colocalization analysis in light microscopy, *J. Microscopy*, 2006, **224**, 213–232.