Polysaccharide-based pH-responsive nanocapsules prepared with bio-orthogonal chemistry and their use as responsive delivery systems

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Supporting information

Experimental section

Materials

All chemicals and materials were used as received if not otherwise mentioned. Rhodamine B isothiocyanate–dextran (MW 10 000, 70 000), tetramethylrhodamine isothiocyanate–dextran (MW 150 000, 500 000) were purchased from Sigma Aldrich. Sulfo-cyanine 5 succinimidyl ester

(Cy5-SE) was purchased from Tocris Bioscience. Polyglycerol polyricinoleate (PGPR) was provided by Danisco and was purified first by dissolution in hexane followed by centrifugation (2000 rpm) to precipitate solid particles, then the supernatant was recover and the purified PGPR was dried by rotary evaporation. Styrene was purified with a column of basic aluminum oxide to remove the inhibitor**.** 2, 2'-Azobis (2-methylbutyronitrile) was recrystallized in ethanol; the resulting crystals were recovered and dried under vacuum and stored until use at -20^{o}C.

Synthesis of nanocapsules precursors

Synthesis of oxidized dextran (OxD)

The oxidation of dextran was carried out by dissolving 5.0 g of dextran (M_n: 16 kDa, $Đ$: 2.18) and 1.75 g of $KClO_4$ in 200 mL of water. The resulting solution was stirred for 24 h at room temperature and then dialyzed for 3 days against water and finally lyophilized (Figure S1). The resulting oxidized dextran was characterized by NMR spectroscopy, FTIR spectroscopy and GPC. The resulting oxidized dextran (M_n: 16 kDa, $Đ$: 2.07) was characterized by NMR spectroscopy, FTIR spectroscopy and GPC.

For *in vitro* cell viability and cell uptake OxD was labeled with cyanine-5. Cy5-OxD was synthesized by reacting 0.5 g of OxD with 6 mg of Cy5-SE, 0.5 mg of 4-dimethylaminopyridine and 7 µL trimethylamine in 50 mL of DMSO. The solution was reacted for 24 h at 50 °C under a nitrogen atmosphere. The resulting solution was dialyzed for 3 days against water and finally lyophilized.

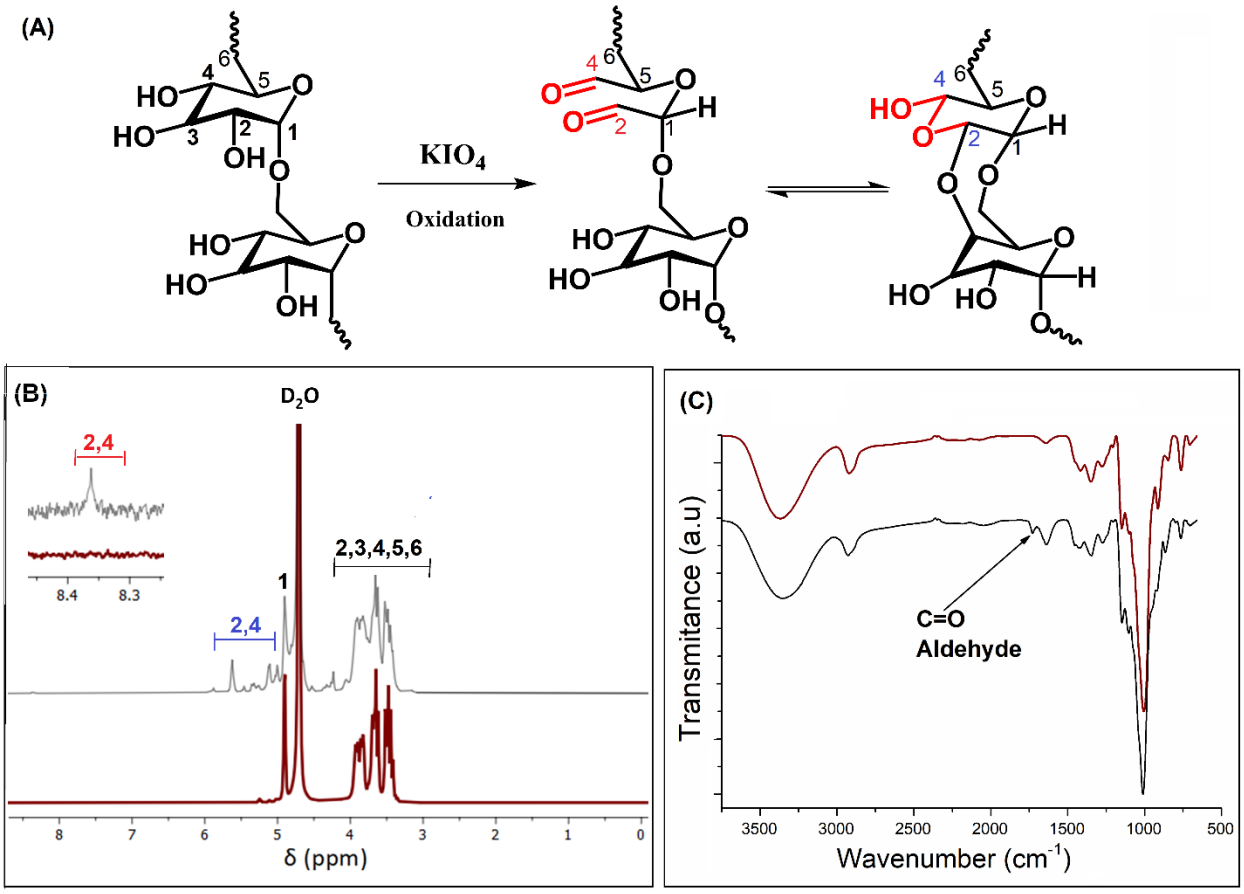

Figure S1. (A) Oxidation of dextran by periodate. (B) ^{1}H-NMR spectrum of native dextran (red) and oxidized dextran (black). (C) FTIR spectrum of native dextran (red) and oxidized dextran (black).

The degree of oxidation (DO) of the OxD defined as the number of oxidized residues per 100 glucose residues was determined by titration. A sample of 0.1g of OxD was dissolved in 25 mL of a 0.25 N hydroxylamine hydrochloride solution in water. Each sample was reacted for 2 h at 50 °C. The solution was then titrated with 0.1 M sodium hydroxide and methyl red. The change of the pH value with the addition sodium hydroxide was recorded to determine the equivalent volume and compared to a blank sample prepared with unreacted dextran. The degree of oxidation measured by titration was 24.7%.

Synthesis of levulinate-functionalized dextran (KeD)

Levulinate dextran was prepared by the reaction of 3 g of dextran (M_n: 16 kDa, $Đ$: 2.18) with 4.30 g of levulinic acid, 7.50 g of DCC, 1.60 g of DMAP and 1.50 g of pyridine in 100 mL of dry DMSO (Figure S2). The solution was reacted for 24 h at 60 °C under a nitrogen atmosphere and then precipitated with ethanol. Then, the precipitate was dissolved in water, filtered through a 200 nm pore size cellulose acetate filter, and then dialyzed over seven days, and freeze-dried. The resulting levulinate dextran (M_n: 18 kDa, $Đ$: 2.12) was characterized by NMR spectroscopy, FTIR spectroscopy and GPC. The degree of functionalization was calculated from the ratio of the NMR peak of the ketone protons of the levulinic acid and the proton on the C1 of the dextran, on average, 100% of the glucose units in KeD were functionalized with one levulinic acid.

For *in vitro* cell viability and cell uptake study KeD was labeled with cyanine-5. Cy5-KeD was synthesized by reacting 0.5 g KeD with 6 mg Cy5-SE, 0.5 mg 4-dimethylaminopyridine and 7 µl in 50 mL of dry DMSO. The solution was reacted for 24 h at 50 °C under a nitrogen atmosphere. The resulting solution was dialyzed for 3 days against water and finally lyophilized.

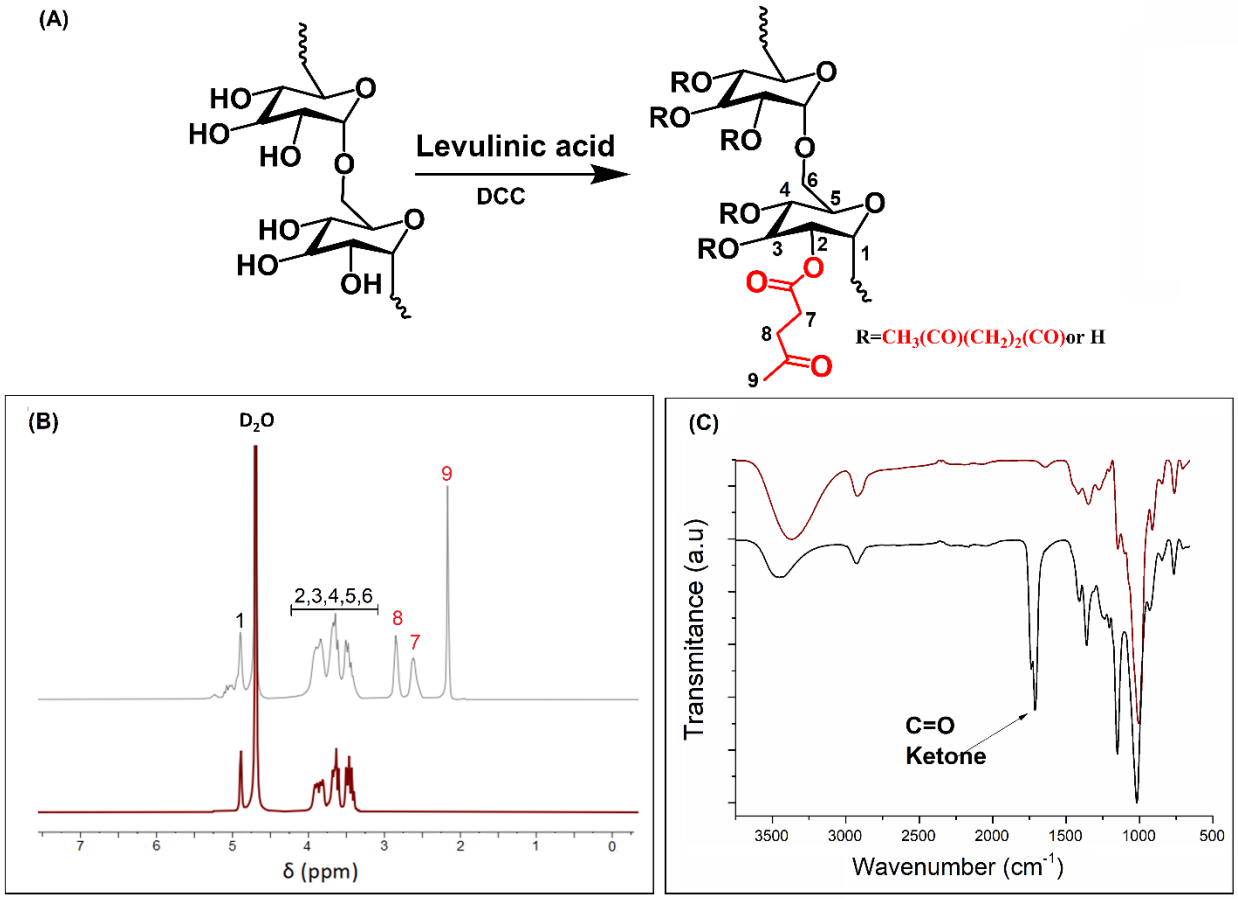

Figure S2. (A) Levulinate dextran preparation. (B) ^{1}H-NMR spectrum of native dextran (red) and levulinate dextran (black). (C): FTIR spectrum of native dextran (red) and levulinate dextran (black).

Synthesis poly(styrene-co-methacryloyl hydrazide) (PSH)

Poly(styrene-*co*-methacryloyl hydrazide) was prepared via a two-step procedure. First, methacryloyl hydrazide was synthesized and then copolymerized by free radical polymerization with styrene to form an oil-soluble hydrazide-containing functional polymer (Figure S3).

Methacrylic anhydride (51.75 g) was dissolved in chloroform (250 mL) and added dropwise to a stirred solution of hydrazine monohydrate (70 mL, 1.44 mol) at 0 °C and then stirred at room temperature overnight. The organic layer was recovered and the aqueous layer washed three times with chloroform. The chloroform aliquots were combined and the solvent removed by rotary

evaporation to yield a white crystalline solid. The resulting solid was recrystallized from a mixture of 10:1 toluene–dichloromethane to yield the pure monomer in the form of fine needle-like crystals (Figure S3). In the second step, the methacryloyl hydrazide (1 g) was copolymerized with styrene (2.4 g) by free-radical polymerization in the presence of AIBN (100 mg) in DMSO at 70 °C overnight. For the polymer purification, water was added to the reaction mixture to precipitate the PSH. The polymer was recovered and dried, redissolved in THF and reprecipitated twice, once in water and once in hexane. The resulting polymer was characterized by NMR, FTIR and GPC (M_n : 1.8 kDa, $Đ$: 1.87).

Titration was used to quantify the hydrazide in PSH. A sample of PSH (50 mg) was dissolved in 20 mL of glacial acetic acid, then one drop of crystal violet indicator was added, and the violet-colored solution was titrated with standard 0.01 N perchloric acid which was prepared in glacial acetic acid and compared to a blank sample prepared without PSH. The endpoint was reached when a definite green coloration was produced. The molar fraction of methacryloyl hydrazide in the polymer was 20.5 mol%.

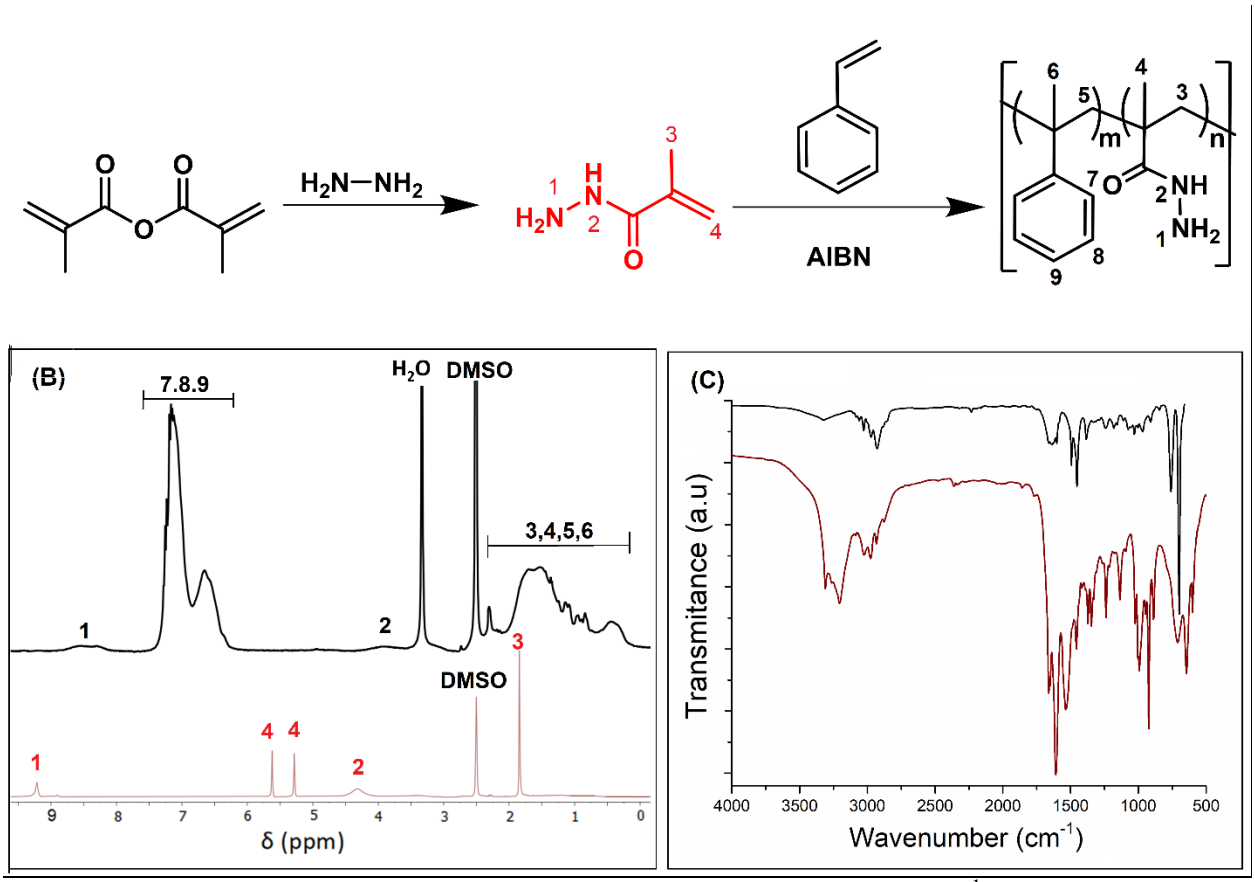

Figure S3. (A) Synthesis of poly(styrene-co-methacryloyl hydrazide). (B) ^{1}H-NMR spectrum of methacryloyl hydrazide (red) and poly(styrene-co-methacryloyl hydrazide) (black). (C) FTIR spectrum of methacryloyl hydrazide (red)) and poly(styrene-co-methacryloyl hydrazide) (black).

Table S1. Properties of the polymers used as nanocarrier precursors.

Polymer	M_n (kDa)	$Đ$	Degree of functionalization (mol%)
Dextran	16	2.18	--
Oxidized dextran (OxD)	16	2.07	25
Levulinate-functionalized dextran (KeD)	18	2.12	100
Poly(styrene-co-methacryloyl hydrazide) (PSH)	1.8	1.87	20

Characterization

The average size and size distribution of the polymer nanocarriers were measured by dynamic light scattering (DLS) at 25 °C using a Malvern NanoS90, working at an angle of 90°. Morphological studies were performed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). For the sample preparation, one drop of diluted nanocarrier suspension was placed onto a silica wafer (for SEM) or a carbon-coated grid (for TEM) and allowed to dry under ambient conditions. The SEM measurements were performed with a 1530 Gemini LEO field emission microscope (Zeiss), with an accelerating voltage of 170 V. For the TEM measurements, a Jeol 1400 transmission electron microscope was used with an accelerating voltage of 120 kV. FT-IR measurements were performed with the Perkin Elmer Spectrum BX FT-IR spectrometer and the spectra were recorded between 4000 and 600 cm^{-1}. For nuclear magnetic resonance (NMR) analysis, ^{1}H and ^{13}C NMR spectra were recorded with Bruker Avance spectrometers operating at a frequency of 300 MHz. Fluorescence intensity measurements were performed on an Infinite M1000 plate reader from Tecan using 96-well plates. The molecular weights of the polymers were measured by gel permeation chromatography (Agilent Technologies 1260 Infinity) equipped with a UV and RI detectors (1260VWD and 1260 RID) and calibrated either with polystyrene standard for analysis in THF or with dextran standards for analysis in water.

HeLa cell culture

The human epithelial cell line HeLa was cultivated with Dulbecco's Modified Eagle Medium (DMEM, Gibco/Thermo Fisher, Germany), supplemented with 10% FBS, 100 U mL^{-1} penicillin, and 100 mg mL^{-1} streptomycin (all Gibco/Thermo Fisher, Germany). The cells were kept in an

incubator at 37 °C, 5% CO_2, and 95% relative humidity (CO_2 Incubator C200, Labotect, Germany) for cultivation.

Cell passaging and harvesting for viability and uptake experiments

The HeLa cells were briefly washed with 7 mL PBS, followed by cell detachment with 7 mL 0.25% Trypsin-EDTA (Gibco/Thermo Fisher, Germany) for 5 min at 37 °C, 5% CO_2, and 95% relative humidity. The cell suspension was transferred with 7 mL FBS supplemented medium and centrifuged at 300g for 5 min (5810R, Eppendorf, Germany). The supernatant was discarded and the cell pellet resuspended in FBS supplemented medium. Cell viability and cell count were determined by equally mixing 20 µL of cell suspension and trypan blue and measuring by an automated cell counter (TC10, Bio-Rad, Germany).

Cell Viability Assay

After cell harvesting, HeLa cells were seeded in a 96-well plate (Item No.: 655083, Greiner Bio-One, Austria) with a cell number of 5,000 cells per well. Following overnight incubation at 37 °C and 5% CO_2, the medium was removed. Nanocarrier dilutions in FBS supplemented DMEM were prepared and added in a volume of 100 µL to the cells. Samples were processed in triplicates. HeLa cells were incubated at 37 °C and 5% CO_2 for 2 h, 24 h, and 48 h before conducting the viability assay. CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Germany) was performed according to the instructions of the manufacturer. The luminescence was measured with an Infinite M1000 plate reader (Tecan, Switzerland).

Cell Uptake Experiment by Flow Cytometry

For cell uptake experiments, HeLa cells were seeded in a 24-well plate after harvesting with a cell number of 150 000 cells per well. Cells were incubated at 37 °C and 5% CO_2 overnight to achieve attachment. On the next day, the medium was removed and the cells washed once with 1 mL of PBS. The nanocarriers were diluted to a concentration of 75 µg mL^{-1} and added in a volume of 200 µL to the washed cells. Samples were processed in triplicates. HeLa cells were incubated at 37 °C and 5% CO_2 for 2 h, 24 h, and 48 h before measuring cell uptake.

Cellular uptake of the nanocarriers was quantified by flow cytometry analysis. After the incubation of the cells with the nanocarriers, the nanocarrier suspension was removed and the cells were washed once with 1 mL of PBS. Then, the cells were detached by adding 250 µL 0.25% Trypsin-EDTA per well, and incubated for 5 min at 37 °C and 5% CO_2. After incubation, 250 µL DMEM with FBS was added and the detached cells were collected in 1.5 mL tubes. The cells were centrifuged at 300 g for 5 min. After centrifugation, the supernatant was discarded and the cell pellet was resuspended in 1 mL of PBS. Flow cytometry measurements were conducted with an Attune™ NxT (Thermo Fisher, Germany). For Cy5 detection, a 638 nm excitation laser was employed with a 670/14 nm band-pass filter. For the detection of the rhodamine-labeled dextran used as the payload a 501 nm excitation laser was used with a 585/16 nm band-pass filter. First, cells were analyzed with FSC/SSC to discriminate cell debris. Subsequently, the gated events of viable cells were analyzed by the fluorescent signal expressed as the median fluorescence intensity (MFI) or as the percentage of gated events. Flow cytometry data analysis was conducted with Attune™ NxT Software (Thermo Fisher, U.S.A).

Visualization of Intracellular Localization by Confocal Laser Scanning Microscopy

For verification of the intracellular localization of nanocarriers, confocal laser scanning microscopy (cLSM) was employed. After harvesting, HeLa cells were seeded in 15 µ-Slide 8 well glass bottom (ibidi) with a cell number of 5 000 cells per well. The cells were incubated at 37 °C and 5% CO_2 overnight to achieve attachment. On the next day, the medium was removed and the cells washed once with 200 µL of PBS. The nanocarriers were diluted to a concentration of 75 µg mL^{-1} and added in a volume of 200 µL to the washed cells. The cells were incubated with this suspension of nanocarriers at 37 °C and 5% CO_2 for 48 h.

Image acquisition was executed on an LSM SP5 STED Leica Laser Scanning Confocal Microscope (Leica, Germany), composed of an inverse fluorescence microscope DMI 6000CS equipped with a multi-laser combination using an HCA PL APO CS2 63 x 1.2 water objective. The Cy5functionalized nanocarriers were excited with a 633 nm laser and detected at 650-750 nm. The rhodamine-labeled payload was excited at 561 nm and detected at 570-620 nm. Lysosomes were stained with LysoTracker Green™ DND-26 (75 nM final concentration, Thermo Fisher, Germany) for 30 min at 37 °C and 5% CO_2 prior to microscopy. Stained lysosomes were excited with a 496 nm excitation laser and detected at 505-550 nm. Plasma membranes were stained with CellMask Green™ (1:1000 diluted, Thermo Fisher, Germany) shortly before microscopy for 5 min in the dark and then imaged using an excitation at 514 nm and detection at 525-550 nm.

Additional results

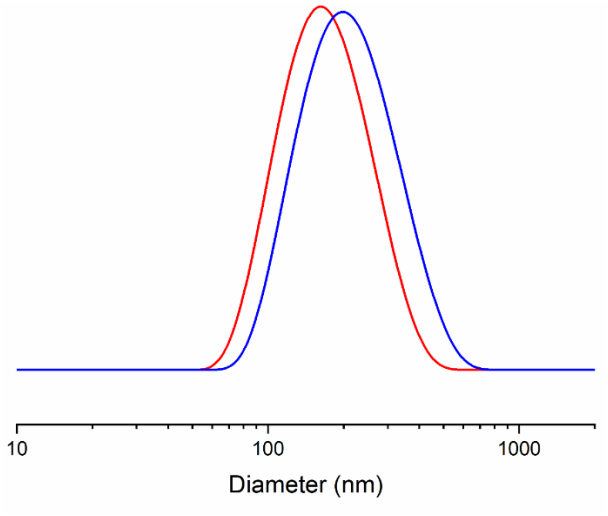

Figure S4. Particle size distribution of dextran NCs in toluene, $OxDNC_{0.5}$ (red line), $KeDNC_{0.5}$ (blue line).

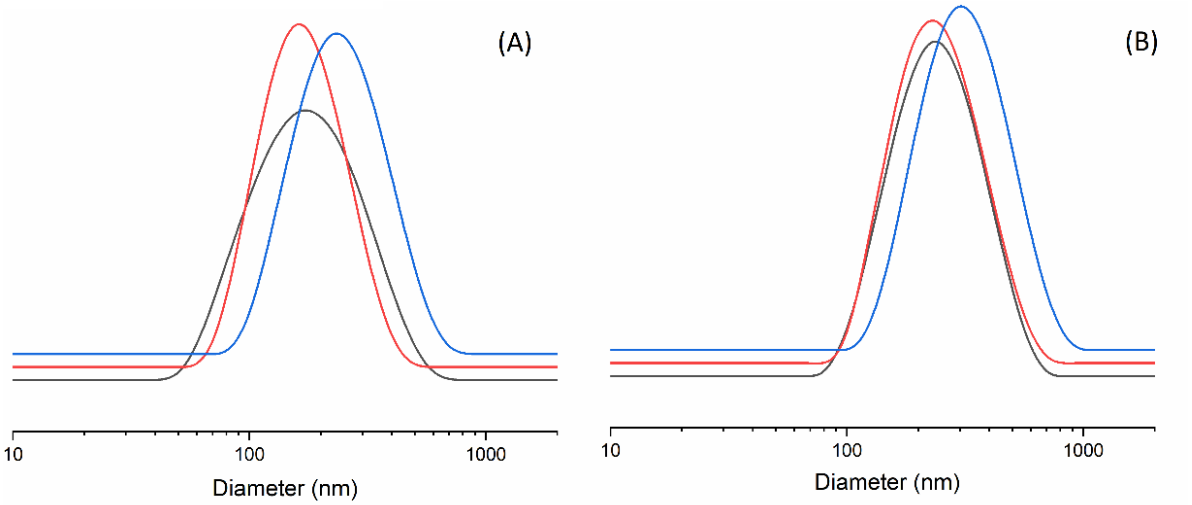

Figure S5. Particle size distribution of the dextran precursor nanodroplets in toluene (black line), of the dextran NCs after crosslinking in toluene (red line), and of the dextran NCs after transfer to water (blue line) for (A) $OxDNC_{0.5}$ (B) $KeDNC_{0.5}$.

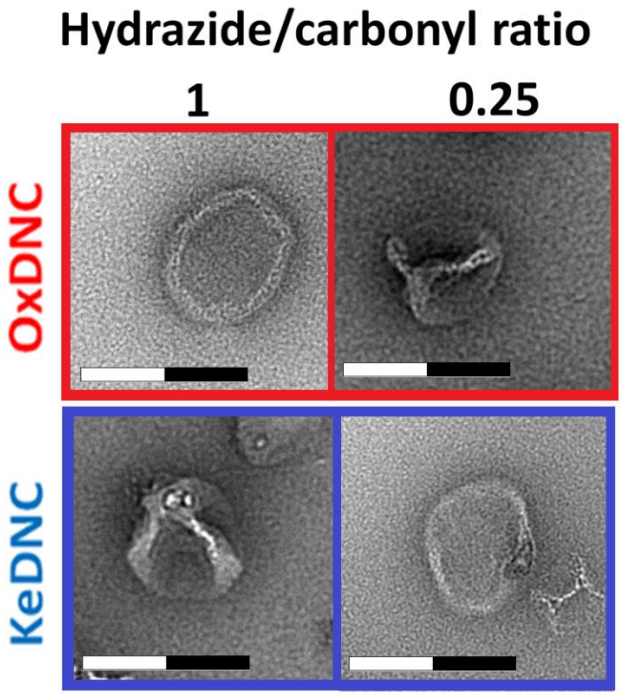

Figure S6. TEM image of the NCs. Scale bars are 200 nm. The thickness of the NCs shell was ca. 10 nm in every case.

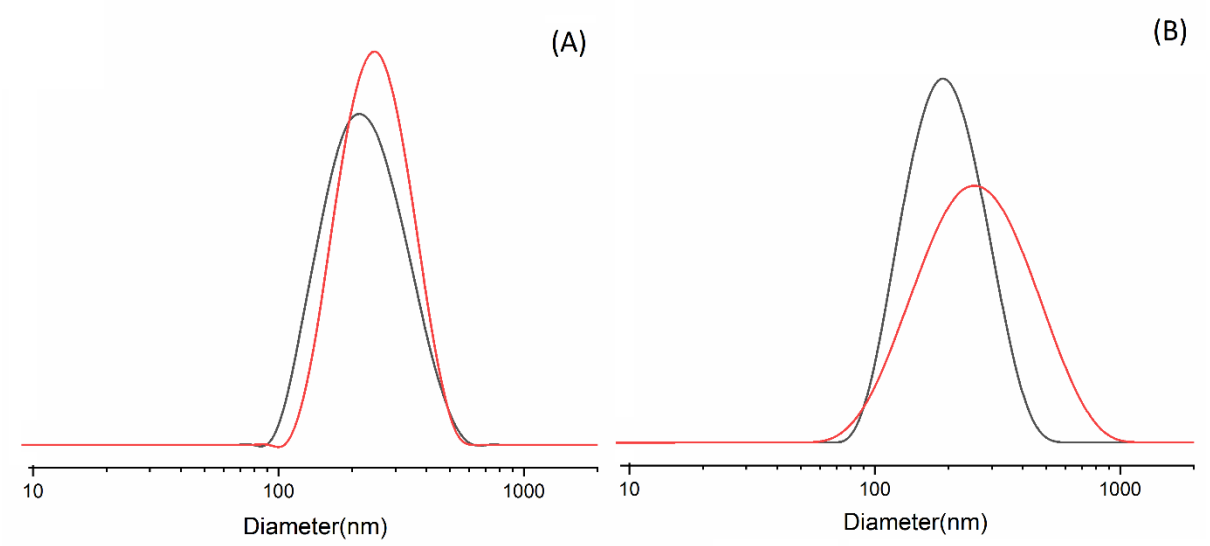

Figure S7. Long term stability of the NCs. Evolution of the particle size distribution of the NCs (A) $OxDNC_{0.5}$ and (B) $KeDNC_{0.5}$, directly after transfer to water (black line) and after 6 months in water (red line).

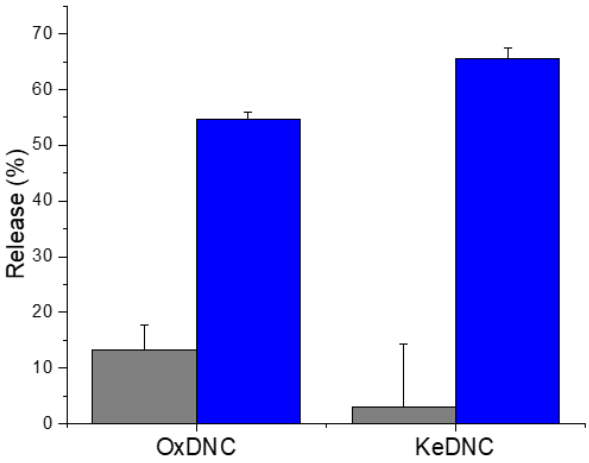

Figure S8. Release of FTIC-albumin triggered by the degradation of the nanocapsule in the presence of dextranase. Release of albumin after incubation for 24 h in suspension with (blue) and without (grey) dextranase.

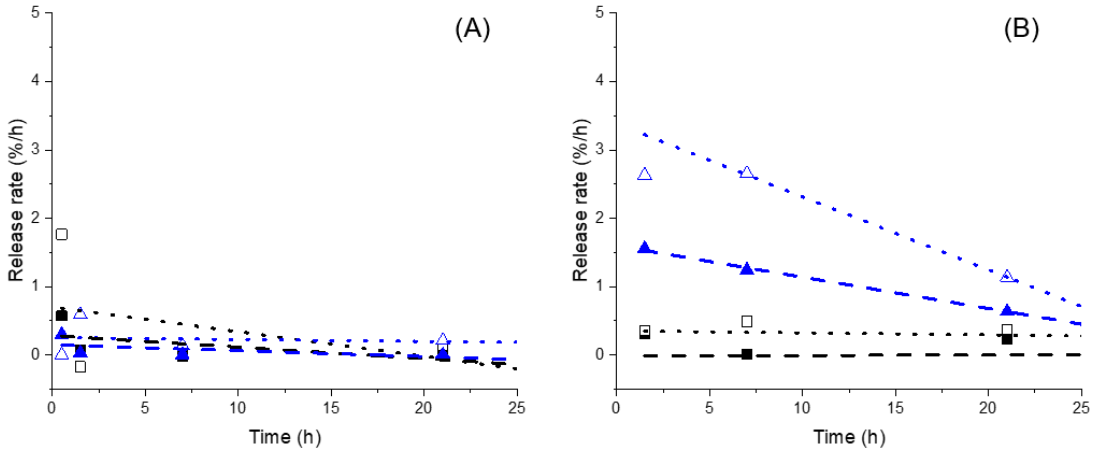

Figure S9. Influence of the payload size and of the pH value of the environment on the release rate of the payloads from (A) OxDNC and (B) KeDNC. For rhodamine-functionalized dextran 150 kDa (▲) and 500 kDa (■) at a pH value of 7.4 (close symbols, dashed lines) and 5.2 (open symbols, dotted lines).

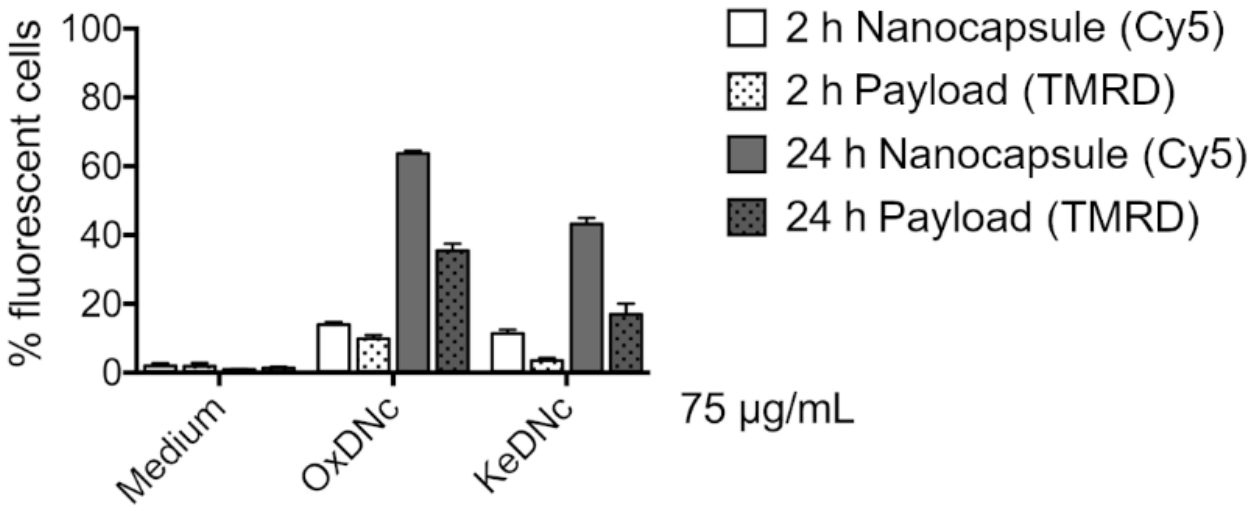

Figure S10. The cellular uptake of the NCs by HeLa cells. The NCs were functionalized with the fluorescent tag cyanine-5 (Cy5) and the payload encapsulated in the NCs (tetramethylrhodamine isothiocyanate-dextran 500 kDa) was labeled with a rhodamine derivative (TMRD).

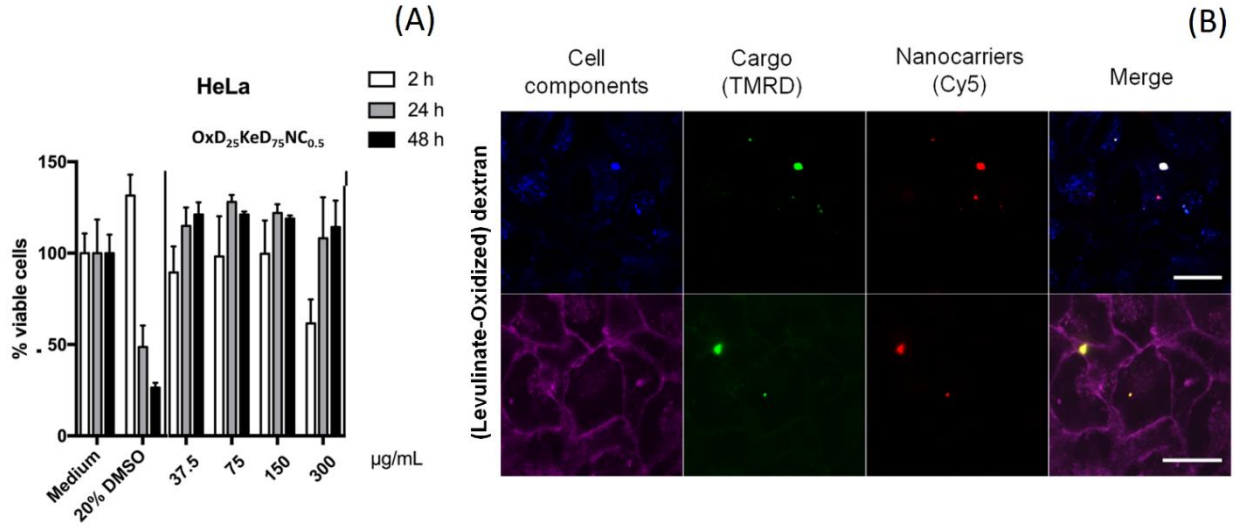

Figure S11. Cellular uptake and cytotoxicity of the mixed functionalized dextran NCs. (A) Cell viability after incubation of HeLa cells with NCs at a concentration ranging from 37.5 to 300 µg/mL for 2, 24 and 48 h. (B) Study of cellular uptake by confocal microscopy after incubation of the HeLa cells for 48 h in media containing 75µg/mL of NCs. The scale bars are 20 µm.