Electronic supplementary information

# Subtle changes in network composition impact the biodistribution and tumor accumulation of nanogels

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## Design strategy of polymers and nanogels

Our aim was to study whether already rather small differences in the chemical composition of polymers that constitute nanogels do result in a different biodistribution of the resulting nanogels. In addition, since that had been shown already for other nanoparticles, we wanted to check the effect of attaching a hydrophilic versus a hydrophobic fluorescence dye.

Accordingly, we have chosen thiolated polyglycidols where the thiolated side chains were either connected with an ester or an ether bond to the polymer backbone as starting material. We on purpose used similar groups, without bigger differences for example in charge or the capacity to form hydrogen bridges, to elucidate whether small changes do result in different biodistribution. Both kinds of polymers were additionally functionalized with either Cy7 or Atto680 fluorophore probe and used for nanogel synthesis. To model the behavior of the disassembled NG after degradation additional quenched free polymer was also tested *in vivo*. Thus four compounds, ester-containing NGs, ether-containing NGs, ester-containing quenched polymer and ether-containing quenched polymer labelled with one of the two fluorescent probes were compared in one set. Influence of the fluorophores could then be seen comparing the two sets.

## Materials

Allyl glycidyl ether (AGE) (99 %, Sigma-Aldrich,St. Louis, MO) dried over calcium hydride (CaH2) (92 %, ABCR, Karlsruhe, Germany) and distilled under reduced pressure. Ethoxy ethyl glycidyl ether (EEGE) was synthesized according to Fitton et al[ESI1]. Phosphate buffered saline was prepared by dissolving the premixed salts (Sigma Aldrich, biograde) in 0.5 L deionized water yielding a 0.01 M solution with a pH 7.4. Borate buffer was made by dissolving sodium tetraborate (Merck, Darmstadt, Germany) in deionized water (0.1 M) and adding chloric acid (0.1 M) to a final concentration of 0.05 M and adjustment of the pH value to 8.5. Acetonitrile (AcN) (HPLC grade, ≥99.9 %) Alloxan monohydrate (98 %), Ammonium salt of 8-anilinonaphthalene-1-sulfonic acid (ANS) (≥97.0 %), 2,2-dimethoxy-2-phenylacetophenon (DMPA)(99 %), 3,3¢-dithiodipropionic acid (DTPA) (99 %),ethyl vinyl ether (99 %, KOH stabilized), glycidol (96 %), 2-hydroxyethyl acrylate (HEA) (96 %), potassiumtert-butoxide (KOtBu) (1 M inTHF), sodiumhydroxide (NaOH) (>98 %), n-hexane (Chromasolv Plus, ≥95 %),p-toluenesulfonic acid monohydrate (pTsOH)(>98.5 %),Span 80, tris(2-carboxyethyl)phosphine hydrochloride(TCEP) (98 %), thioacetic acid (96%), trifluoroacetic acid (TFA), Tween 80 were purchased from Sigma-Aldrich (St. Louis, MO) and used as received. Atto680-maleimid (AttoTec, Siegen, Germany), deuterated chloroform ($CDCl_3$) (Deutero GmbH, Kastellaun, Germany); Cy7-allyl (custom made, Lumiprobe, Hannover, Germany) Deuterium oxide ($D_2O$) (Deutero GmbH, Kastellaun, Germany), diethylether (Staub Co., Nuernberg, Germany), DPBS (Gibco, Thermo Fisher Life technologies, Darmstadt, Germany),ethanol (EtOH) (99%, TH Geyer, Renningen, Germany), hydrochloric acid (HCl) (32%, Merck, Darmstadt, Germany), 2-hydroxy-1-[4-(hydroxyethoxy)-phenyl]-2-methyl-1-propanone (Ir-gacure 2959) (BASF, Ludwigshafen, Germany), tetrahydrofuran (THF) (Fisher Scientific, Schwerte, Germany),

sodium hydrogen carbonate ($NaHCO_3$)(Merck, Darmstadt, Germany) and sodium sulfate ($Na_2SO_4$) (Merck, Darmstadt, Germany) were used as received.

## Characterization

$^1$H NMR spectra were recorded on a 300 MHz spectrometer (Bruker NMR Fourier 300) and a 600 MHz spectrometer (Bruker Avance III HD 600) using $CDCl_3$ or $D_2O$ as a solvent. Dynamic light scattering (DLS) measurements in solution were performed on a Zetasizer NanoZSP system (Malvern, UK). Particle size and concentration was determined with the nanoparticle tracking analysis (NTA) device NS500 (Nanosight/Malvern, UK). High pressure liquid chromatography (HPLC) was performed on a Shimadzu device (components: LC-20 AT Series-type double plunger, SIL-20AC Autosampler, RF-20A Fluorescence Detector, SPD-M20A Photodiode Array Detector. CTO-20AC Column Oven, CBM-20A System Controller, RID-20A Refractive Index Detector). Fluorescence intensity of the dyes was read on a Spark 20M microplate reader (TECAN; Switzerland). Fluorescence spectra were recorded using a Photon Technology International Fluorescence spectrometer, (components: QuantaMasterTM Model QM-2000-4, cooled photomultiplier (type R928P), InGaAs-NIR detector, 75 W xenon short arc lamp (type UXL-75XE, Ushio). For in vivo imaging, a fluorescence Diffuse Optical Tomography (fDOT) imaging system developed by CEA/LETI and Cyberstar[ESI2] was used. This imaging system is composed of near-infrared LEDs and lasers for illumination, and a Charge-Coupled Device (CCD) camera and two filter sets for detection. The CCD camera is focused at the top surface of the animal. This instrument acquires planar and 3-dimensional images. The acquisition of planar images is based on the excitation of fluorophores by the LEDs (emitting light between 650 and 670 nm) placed above the animal and on the reception of the fluorescence signal using the CCD camera and a band-pass filter ($730 \pm 15$ nm) or a long-pass filter (750 nm). Before acquisition, the exposition/acquisition time is specified.

## Synthesis of thiolated polyglycidol

Thiol functionalized ester-free poly(glycidol) was synthesized as described previously in Stichler et. al[ESI3]. Briefly poly(glycidylethylether)-stat-poly(allylglycidylether) was synthesized via anionic polymerization and deprotected under acidic conditions in THF (SEC (DMF): Mn = 5500 Da, Đ = 1.27 ). Thioacetic acid was attached using thiol-ene click chemistry in ethanol with DMPA as photoinitiator and irradiation at 360 nm for 2 hours. Subsequent basic deprotection with NaOH at 70°C for 3 h and reduction with TCEP HCl (0.5 eq. with respect to thiol units) at room temperature was performed. After purification via dialysis against ultra-pure water (MWCO 3500 dialysis membrane) for 3 days under Argon, the polymer solution was lyophilized and the resulting polymer PGSH EF was stored at -20°C under argon for further use. 1H NMR showed a complete conversion from allyl groups to thiol groups (14 % SH functionality). $^1$H NMR (300 MHz, $D_2O$, δ): 3.93–3.37 (m, 341 H, backbone-H), 2.72–2.51 (m, 17 H, –O–CH2–CH2–CH2–SH), 1.97–1.77 (m, 15 H, –O–CH2–CH2–CH2–SH), 1.22 (s, 9 H,tBu-H) ppm.

Thiol functionalized ester-containing poly(glycidol) was synthesized according to Stichler et. al.[ESI3]. In short the pre-polymer poly(glycidol) was synthesized via anionic polymerization of ethoxy ethyl glycidol ether and deprotection under acidic conditions

(SEC (DMF): Mn = 5200 Da, Đ = 1.23). The polymer was crosslinked by Steglich esterification with 3,3'-dithiopropionic acid aiming for 20 % of functionalisation with respect to hydroxyl groups. The obtained gel was reduced in aqueous solution with TCEP HCl and a pH of 7 adjusted with trimethylamine. Dialysis was performed against ultra-pure water (MWCO 3500 dialysis membrane) under argon for 3 days. Finally the thiolated ester-containing poly(glycidol) PGSH EC was obtained after lyophilisation and stored at -20 °C until further used. 1H NMR showed 13.5 %-SH functionality. $^{1}$H NMR (300 MHz, $D_2O$, δ): 4.48–4.15 (m, 19H,–CH2–OOC–CH2–CH2–SH), 3.93–3.35 (m, 332 H, backbone-H), 2.86–2.70 (m, 33 H, –CH2–OOC–CH2–CH2–SH), 1.22 (s, 9 H, tBu-H) ppm.

## Functionalization of thiolated polyglycidol with dyes

$PG\text{-}SH_{EC}$ or $PG\text{-}SH_{EF}$ was dissolved in PBS buffer to a final concentration of 10 mg $mL^{-1}$, degassed in the ultrasonic bath for 5 minutes and another 20 minutes with argon. Atto680 (2.3 µmol, labelling of every 16.9 polymer chain) was dissolved in 400 µL anhydrous DMSO. The dye solution was added to the polymer solution and stirred for 2 h at RT under argon. The solution was dialyzed using a 3500 MWCO dialysis membrane (dry storage) in degassed deionized water (2 h Ar flow/2 L water) for 3 days (3 changes/day). The solution was centrifuged; the upper phase was transferred to a flask, degassed 10 minutes and lyophilized. The product was stored under argon at -20°C until further use.

$PG\text{-}SH_{EC}$ or $PG\text{-}SH_{EF}$ respectively was dissolved in anhydrous DMSO to a final concentration of 34.2 mg $mL^{-1}$, degassed 30 minutes with Ar and DMPA (10 mg, 39.02 µmol) was added. Cy7-allyl (13.60 µmol PGSH EF and PGSH EC 13.11 µmol respectively labelling of every 3rd polymer chain) was dissolved in 100 µL anhydrous DMSO. The dye solution was added to the polymer solution and stirred at room temperature under UV irradiation (365 nm, UV LEDs, 4911 W, Polymerschmiede, Aachen, Germany) for 2 min. The solution was diluted by addition of 60 mL deionized water and dialyzed using a 3500 MWCO dialysis membrane (dry storage) in degassed deionized water (2 h Ar flow/2 L water) for 3 days (3 changes/day). The solution was centrifuged; the upper phase was transferred to a flask, degassed 10 minutes and lyophilized. The product was stored under argon at -20°C until further use.

## CAC estimation nanoparticle tracking analysis

Measurements were performed using NTA. Polymer solution of various concentrations (2 mg $ml^{-1}$ to 0.025 mg $mL^{-1}$) was prepared. Measurements were carried out in triplicate measuring every sample three to five times with a camera level of 16 and analyzed with a threshold of 8 using automated settings. The particle concentration and size distribution of the sample was calculated as a mean value over all measurements.

## CAC estimation via fluorescence spectrophotometry

Ammonium salt of 8-anilinonaphthalene-1-sulfonic acid (ANS) was dissolved in deionized water and added to a polymer solution of various concentrations (2 mg ml to 0.025

mg $mL^{-1}$) yielding a final concentration of 5µM Samples containing ANS were stirred at room temperature for 3 h and the fluorescence emission spectra were recorded between 400 nm and 600 nm (bandwidth 5 nm) at an excitation wavelength of 370 nm (bandwidth 5 nm). Measurements were performed in triplicate.

## Nanogel preparation

Thiolated fluorescently labelled poly(glycidol) was dissolved in 62.5 µL borate buffer yielding a concentration of 0.4 mg $ml^{-1}$. Tween 80 and Span 80 (3.5 µmol and 0.33 µmol) were dissolved in n-hexane keeping the ratio between aqueous and organic phase 1:10. The dispersion was sonicated using BRANSON SONIFIER 250 Digital with a microtip at an intensity of 10 % a sonication length of 0.4 s and a pause of 0.6 s. The first sonication was applied for 60 s then 30 µL of a freshly prepared aqueous solution of alloxan (0.2 M) was added and the dispersion was sonicated another 60 s. The emulsion was stirred for 20 minutes and HEA (26.12 mM in 1 mL of PBS) was added to the emulsion for quenching. After additional 20 minutes of stirring, the emulsion was centrifuged 1 minutes at 14000 rpm to induce phase separation. The upper phase was discarded, fresh n-hexane was added and the mixture was homogenized. The washing procedure was repeated one more time with hexane and once with THF. The nanogel dispersion was dialysed in deionized water (12-14000 MWCO dialysis membrane). The nanogel solution was then stored at 4°C for further use.

## Preparation of quenched pre-polymers

Thiolated fluorescently labelled poly(glycidol) was dissolved in borate buffer (20mg $ml^{-1}$) and 1 mL of of an aqueous HEA solution (0.51 M) was added. The solution was stirred for 1 h and dialyzed against deionized water yielding the final quenched pre-polymer solution.

## Determination of particle size distribution and particle concentration

Measurements were performed via NTA. Samples were diluted in a ratio of 1:10000 in a total volume of 1 mL ultra-pure water. Each sample was measured three to five times with a camera level of 16 and analyzed with a threshold of four to five using automated settings. The particle concentration and size distribution of the sample was calculated as a mean value over all measurements.

## Degradation study

Nanogels were prepared from PGSH EF and PGSH EC respectively in triplicate. Particle concentration was adjusted to $1.02\times10^{12}$ particles $mL^{-1}$ for all samples and dispersed in water or DPBS. Particles were incubated at 37°C for 8 days. Samples were taken every day and particle concentration and size distribution were determined via NTA.

## DLS measurements of polymers in bovine serum

All polymers, labelled and non-labelled, were dissolved in DPBS (2 mg $ml^{-1}$). The polymer solution was added to FCS yielding a 92 vol % FCS solution as calculated for the theoretic ratio of mouse blood to injected polymer solution. As a control FCS with added DPBS and pure serum were measured. All samples were incubated at 37°C and measurements were performed at 37°C. All samples were measured in triplicates and mean values were calculated.

## Cytotoxicity assays

5·104 cells/well using the HFF cell line were seeded in a 24-well plate. After 3 h the medium was changed and nanogels were added.

Cell viability of $NG_{EF}$ and $NG_{EC}$ was assessed by CellTiter-Glo Luminescent Cell Viability Assay after 48 h. Luminescence was recorded and background luminescence was subtracted. The data represents the average of 3 independent replicates, normalized to the untreated sample.

Cell number was determined using Casy TT cell counter (Omni Life Science, Bremen) after 48 h.

**In-vivo studies**

**Ethics Statement**

All animal use procedures for subcutaneous MDA-MB-231 tumour models and in vivo fluorescence imaging were in strict accordance with the recommendations of the European Community (86/609/CEE) and the French National Committee (décret 87/848) for the care and use of laboratory animals. Ethics committee of CETEA – CEA DSV (Comite d'Ethique en Expérimentation Animale (CETEA), de la Direction des Sciences du Vivant (DSV) du Commissariat à l'Energie Atomique et aux énergies alternatives (CEA)) approved the study (ref: 12-093).

**Animal model**

Female nude mice were purchased at JANVIER LABS (Saint-Berthevin, France). They were housed under standard conditions with food and water ad libitum until they weighted ~25 gr. MDA-MB231 cancer cells, purchased from ATCC (Manassas, VA), were subcutaneously implanted in the dorsal fat pad. More specifically, $10^6$ cells were injected in a 100 µL volume of PBS (Phosphate Buffer Saline) with 100 µL of Matrigel (BD Bioscience, Le Pont de Claix, France) at 0°C.

## Biodistribution evaluation of ester-free and ester-containing Cy7-labelled nanogels and polymers in vivo

A total of three mice or four mice per group received intravenous injections of each Cy 7 or Atto680 – labelled compound (150 µL of each at a concentration described in table S1). Whole body planar NIR-imaging was performed using the planar imaging option of the TomoFluo3D fluorescent tomographic system (developed by CEA/LETI and Cyberstar)[2]. First, whole body planar images of dorsal and ventral side views of mice before injection were acquired in order to record the autofluorescence of each animal. After injecting the animals with each compound, images were obtained at different times post-injection, over a period of 72h. These images provided general information about the biodistribution, elimination pathways and tumor targeting of the different compounds. Another assessment of the distribution of the Cy 7 and Atto680 - labelled compounds was implemented, by *ex vivo* planar NIR-imaging of the organs, harvested 72 h post i.v. injection. The fluorescence values of every organ were subtracted by its autofluorescence signal measured from a non-injected mouse.

## Supplementary Figures and Tables

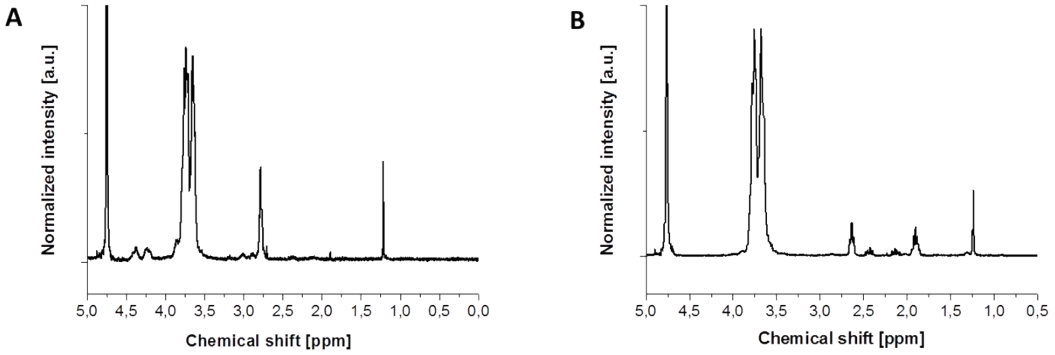


**Figure S 1**. $^{1}H$ NMR (300 MHz, $D_2O$) of (A) PG-$SH_{EC}$ and (B) PG-$SH_{EF}$.

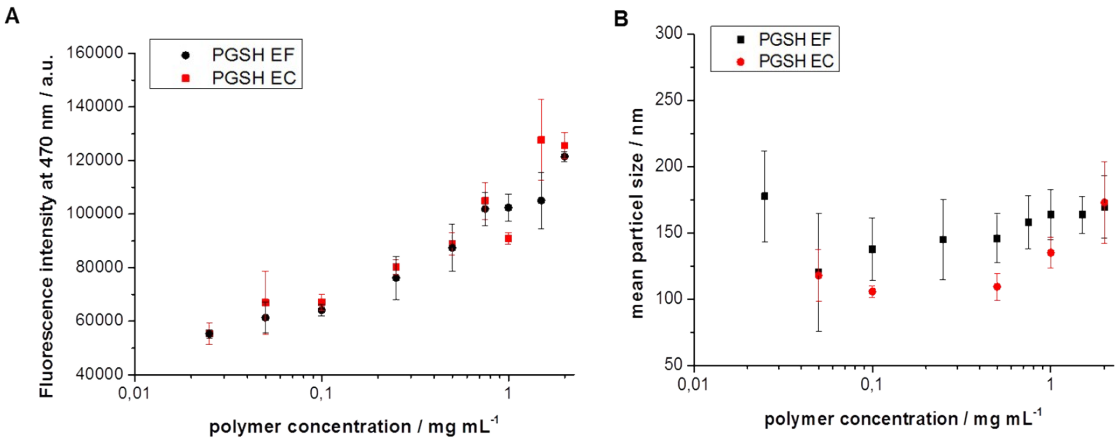


**Figure S 2**. (A) Fluorescence intensity of ANS at 470 nm at different polymer concentrations for determination of critical aggregate concentration (average value ± SD(n = 3)). (B) Aggregate size of PG-$SH_{EC}$ and PG-$SH_{EF}$ in dependence of polymer concentration measured with NTA in water (average value ± SD (n = 3))

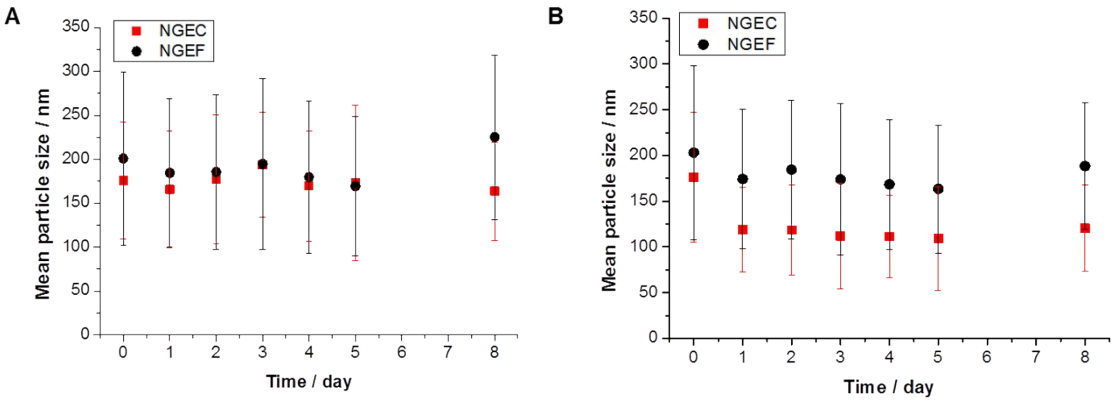


**Figure S 3.** Degradation study of $NG_{EC}$ and $NG_{EF}$ in (A) water and (B) DPBS at 37° during 8 days determined by NTA size measurements (average value ± SD(n = 3)).

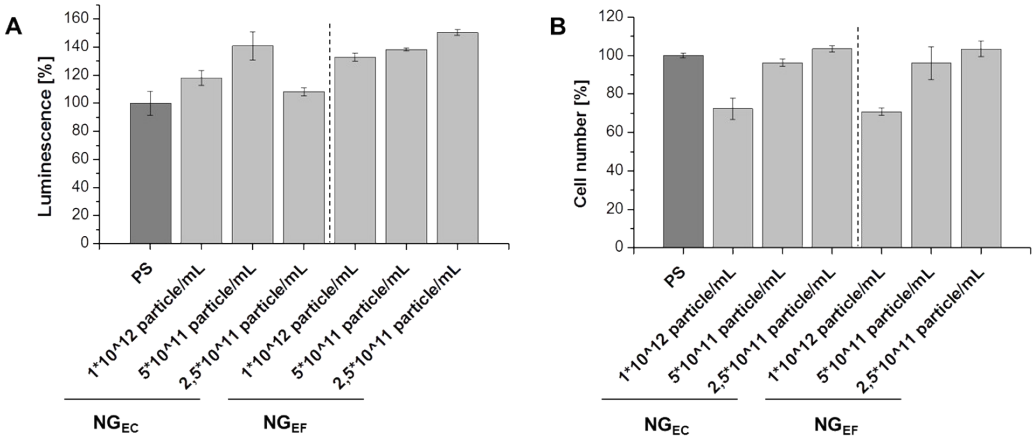


**Figure S 4.** Viability of HFF cells determined via luminescence assay. (A) Luminescence and (B) cell number after 48 h of incubation with $NG_{EC}$ and $NG_{EF}$ at different concentrations in percentage relative to the control.

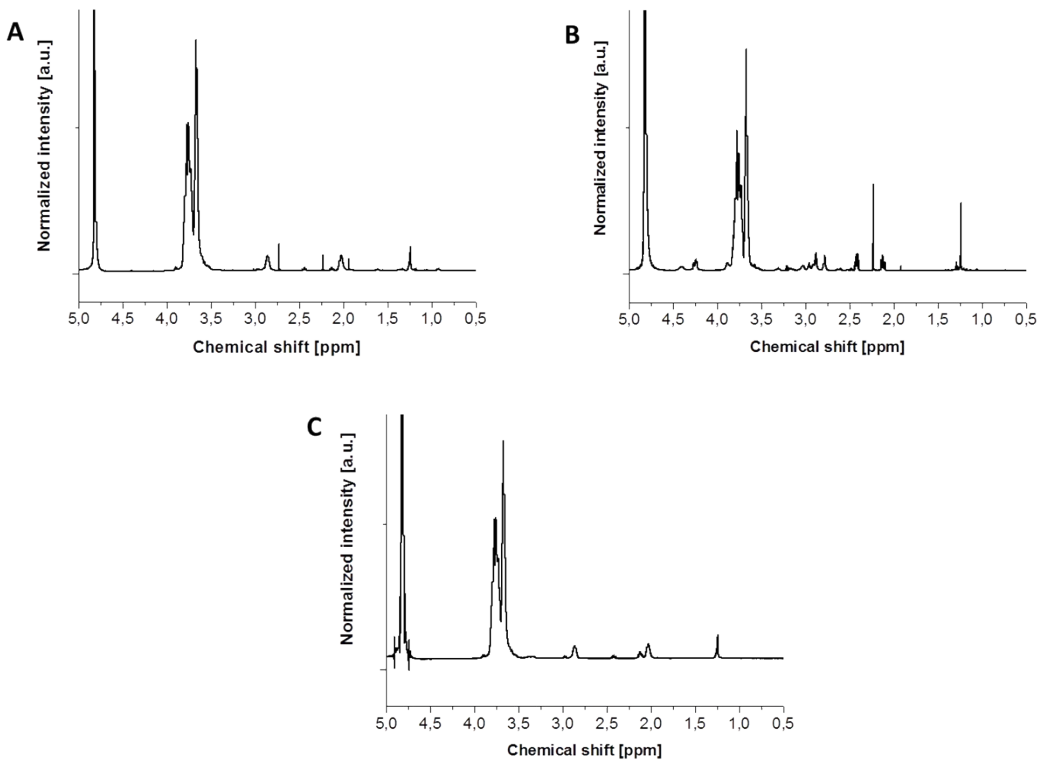


**Figure S 5.** $^{1}$H NMR (600 MHz, $D_2O$) spectra of (A) Atto680-labelled $PGSH_{EF}$, (B) Atto680-labelled $PGSH_{EC}$ and (C) Cy7-labelled $PGSH_{EF}$.

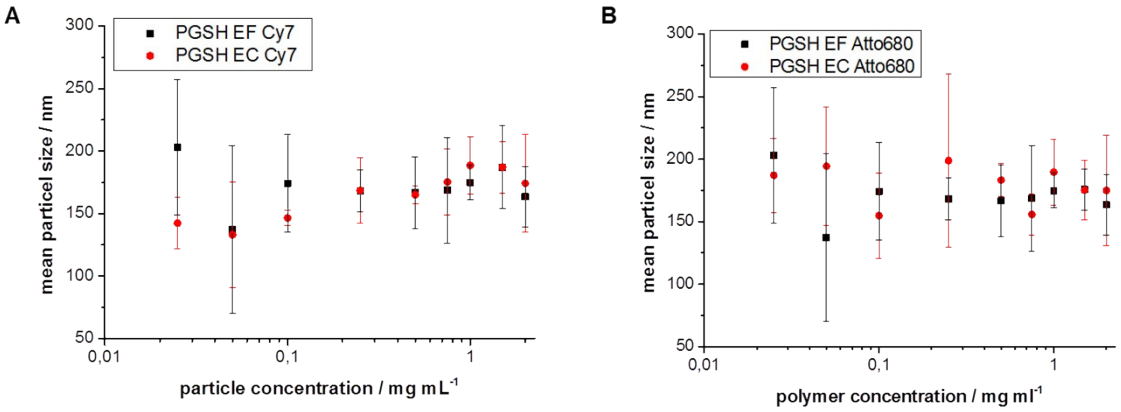


**Figure S 6.** (A) Aggregate size of Cy7- and (B) Atto680-labelled $PGSH_{EC}$ and $_{EF}$ in dependence of polymer concentration measured with NTA in water.

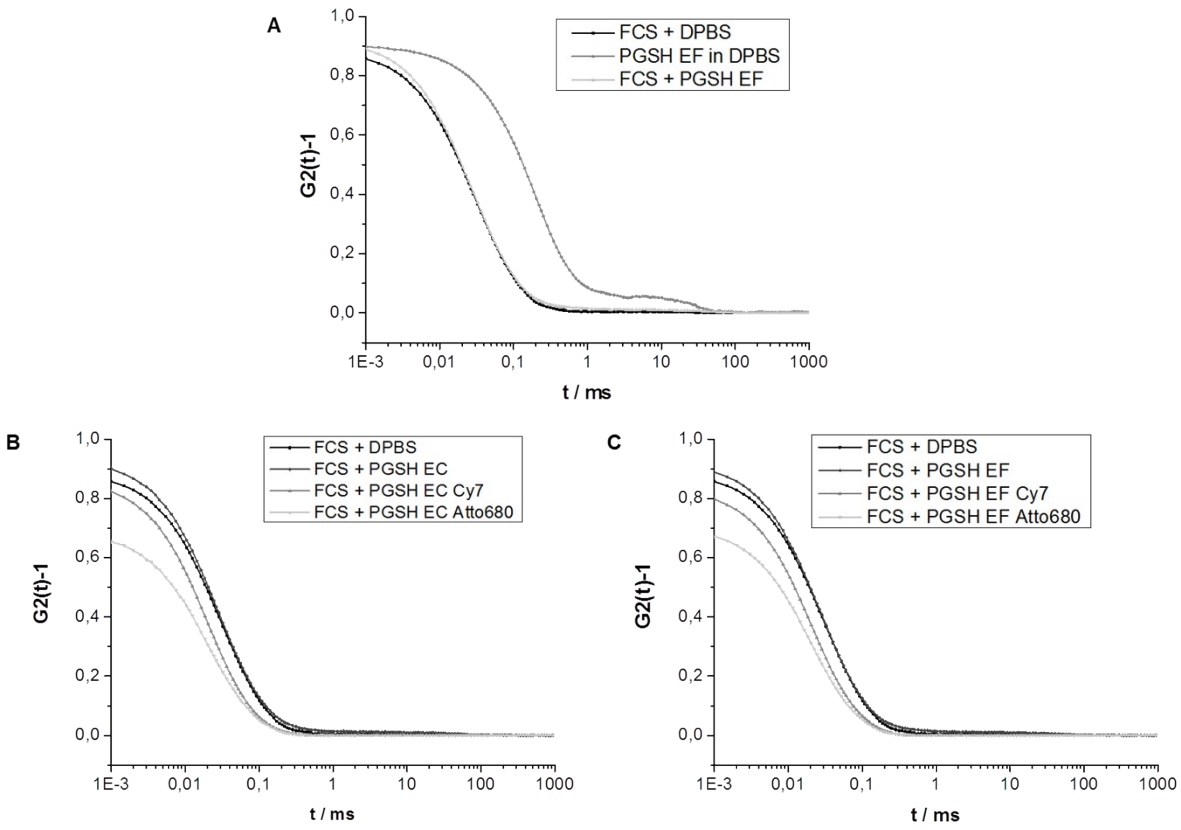


**Figure S 7.** Study of serum protein interaction with non-labelled as well as labelled and quenched $PGSH_{EF}$ and $_{EC}$ monitored via DLS at 37°C. Normalized intensity autocorrelation functions of (A) FCS diluted with DPBS to 92 vol%, $PGSH_{EF}$ in DPBS and $PGSH_{EF}$ (2 mg $ml^{-1}$ in DPBS) added to FCS yielding 92 vol%, (B) FCS diluted with DPBS to 92 vol% in comparison with $PGSH_{EC}$, $PGSH_{EC}$ Cy7 and $PGSH_{EC}$ Atto680 (2 mg $ml^{-1}$ in DPBS) added to FCS yielding 92 vol. (C) FCS diluted with DPBS to 92 vol% in comparison with $PGSH_{EF}$, $PGSH_{EF}$ Cy7 and $PGSH_{EF}$ Atto680 (2 mg $ml^{-1}$ in DPBS) added to FCS yielding 92 vol%. (Intensity shift of labelled polymers in (B) and (C) due to interactions of the fluorophores with the laser light of the DLS device)

**Table S 1.** Characteristics of Cy7-labelled compounds used for *in vivo* studies concerning particle size, concentration and degree of fluorescent labelling.

| | **Particle concentration** **± SD [particle $mL^{-1}$]** | **Mean particle diameter** **± SD [nm]** | **Degree of fluorescent labelling** | **Mass concentration** **± SD [mg/mL]** |
|---|---|---|---|---|
| **NGEC Cy7** | $1.30\times10^{12}$ ± $1.04\times10^{11}$ | 200 ±108 | | 3,40± 0,00 |
| **NGEF Cy7** | $1.31\times10^{12}$ ± $8.33\times10^{11}$ | 218 ±90 | | 2,60± 0,57 |
| **PGSHq EC Cy7** | - | - | 7% of polymers labelled | 5,50± 1,98 |
| **PGSHq EF Cy7** | - | - | 17% of polymers labelled | 5,80± 2,12 |
| **NG EC Atto680** | $4.38\times10^{12}$ ± $7.15\times10^{11}$ | 234 ± 96 | | 4,70 ± 0,14 |
| **NG EF Atto680** | $3.28\times10^{12}$ ± $6.32\times10^{11}$ | 279 ± 122 | | 2,10 ± 0,42 |
| **PGSHq EC Atto680** | - | - | 9% of polymers labelled | 3,70 ± 0,71 |
| **PGSHq EF Atto680** | - | - | 10% of polymers labelled | 4,10 ± 0,42 |

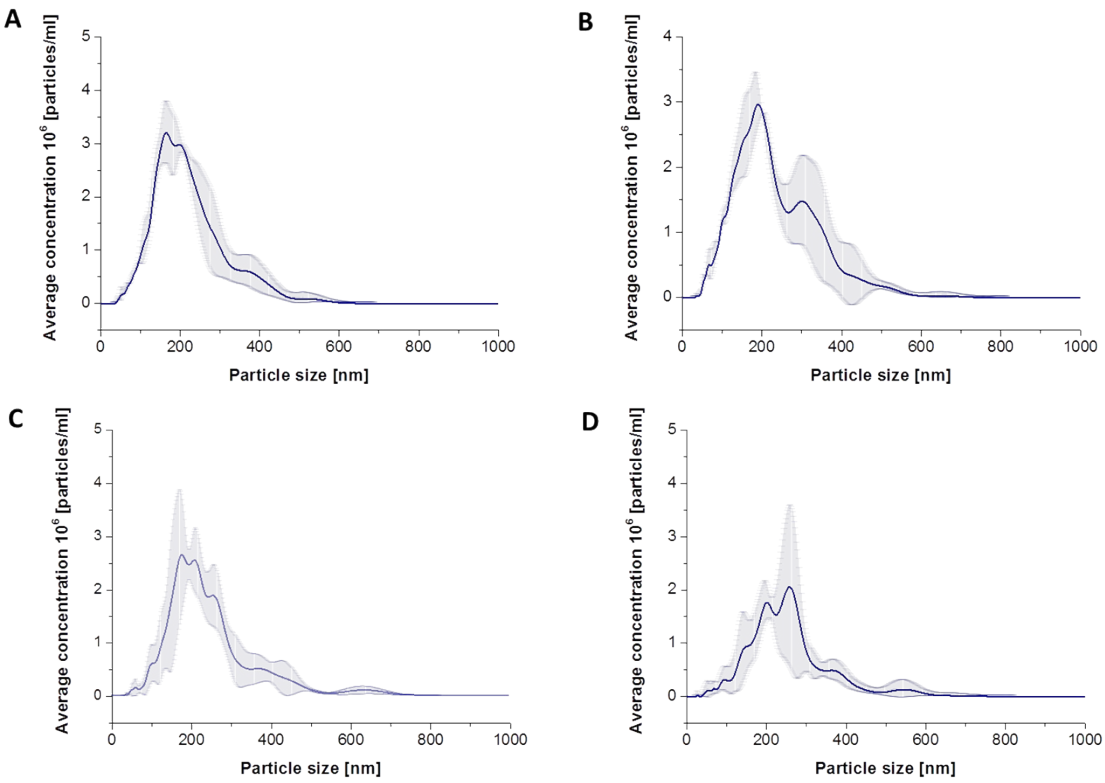


**Figure S 8.** Particle size distribution of (A) NGEC Cy7, (B) NGEF Cy7, (C) NGEC Atto680 and (D) NGEF Atto680 measured by nanoparticle tracking analysis (NTA). Mean particle diameter shown in Table S1.

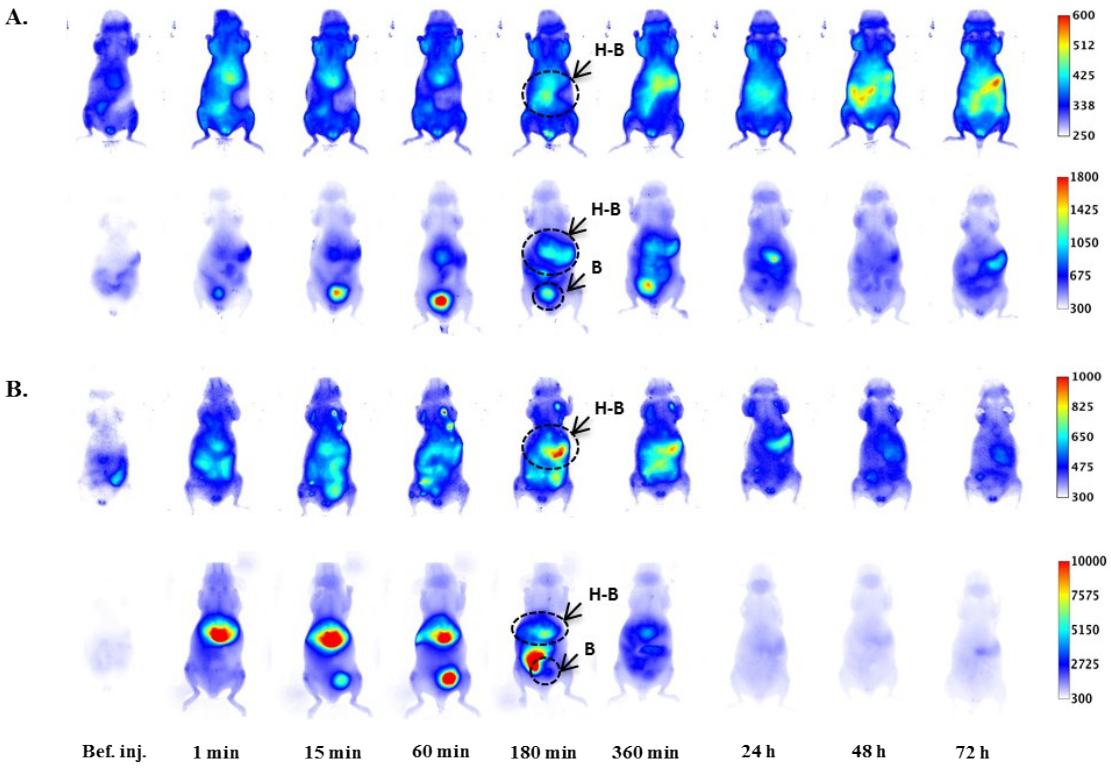


**Figure S 9.** Ventral side view whole body NIR fluorescence imaging of mice injected with (A) $NG_{EF}$ Cy7 (upper row), PG-$SHq_{EF}$ Cy7 (bottom row) and (B) $NG_{EC}$ Cy7 (upper row), PG-$SHq_{EC}$ Cy7 (bottom row). Dashed regions and arrows illustrate elimination organs such as liver and intestines (H-B) and bladder (B). Attention should be given to the color scales.

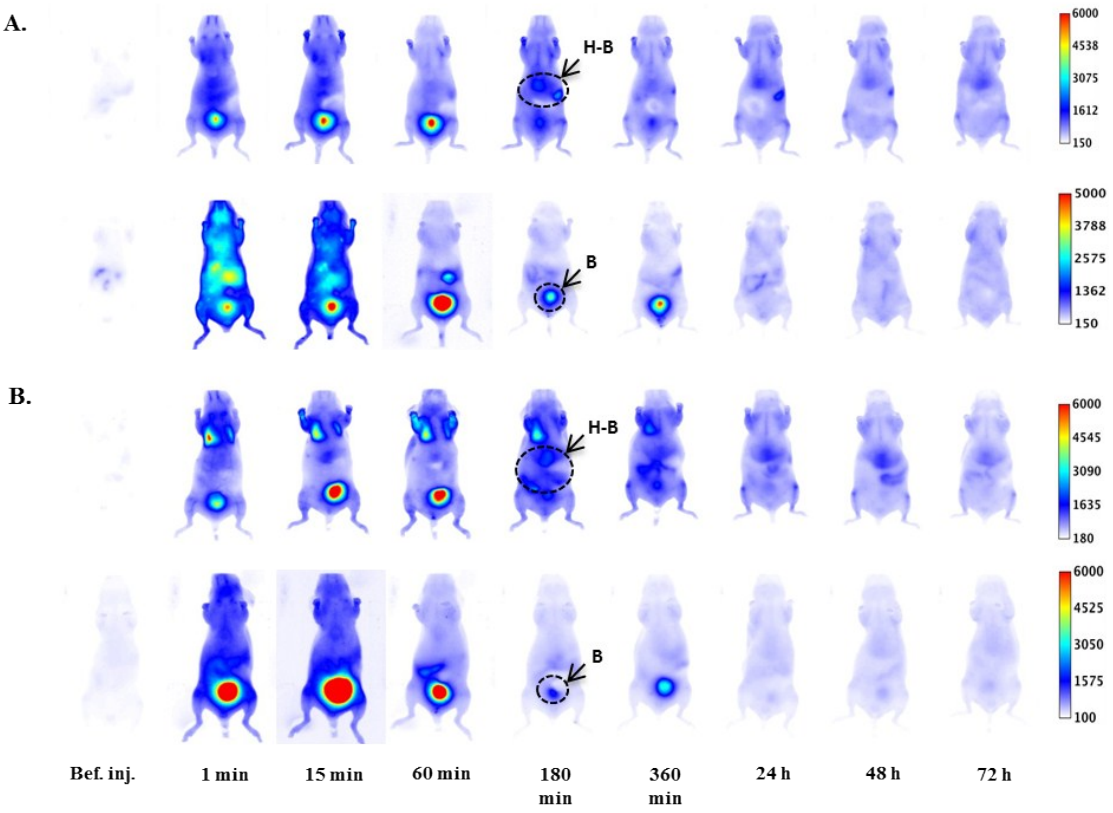


**Figure S 10.** Ventral side view whole body NIR fluorescence imaging of mice injected with (A) $NG_{EF}$ Atto680 (upper row), PG-$SHq_{EF}$ Atto680 (bottom row) and (B) $NG_{EC}$ Atto680 (upper row), PG-$SHq_{EC}$ Atto680 (bottom row). Dashed regions and arrows illustrate elimination organs such as liver and intestines (H-B) and bladder (B). Attention should be given to the color scales.

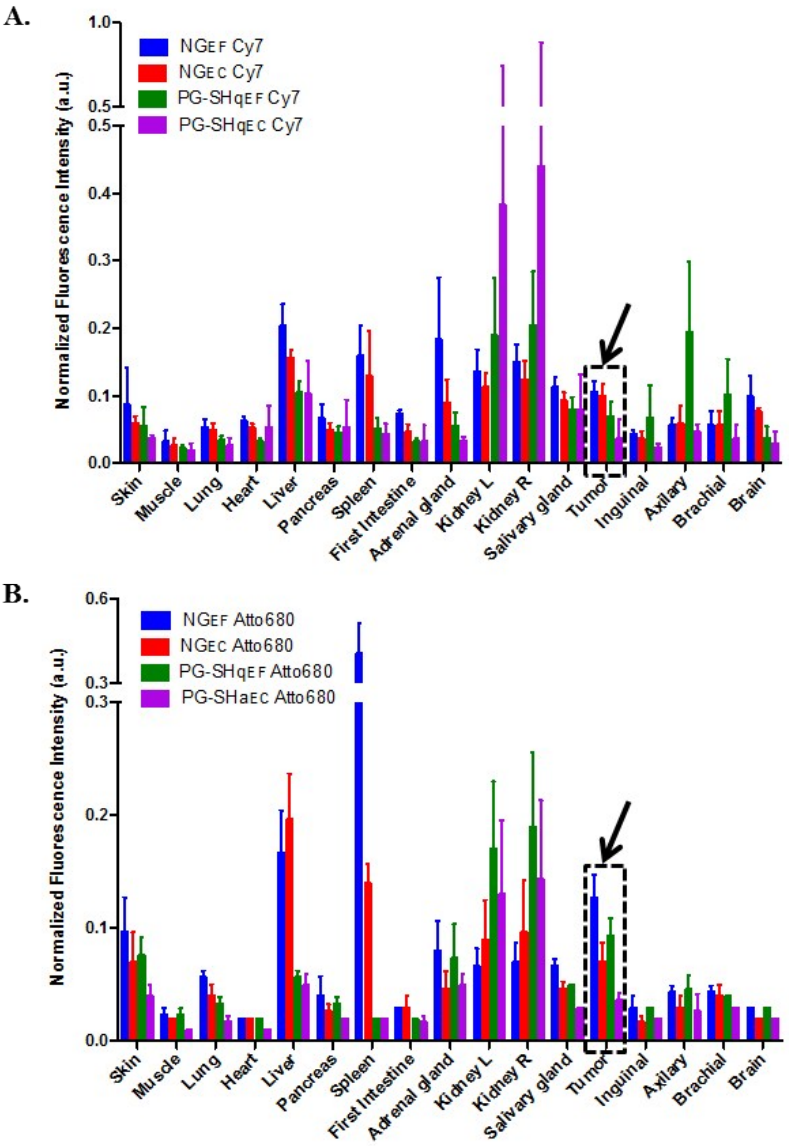


**Figure S 11.** *Ex vivo* organ and tumor fluorescence intensity (subtracted by their autofluorescence signal) 72 h after injection of (A) Cy7-labelled and (B) Atto680-labelled compounds (average value ± SD (n = 3).

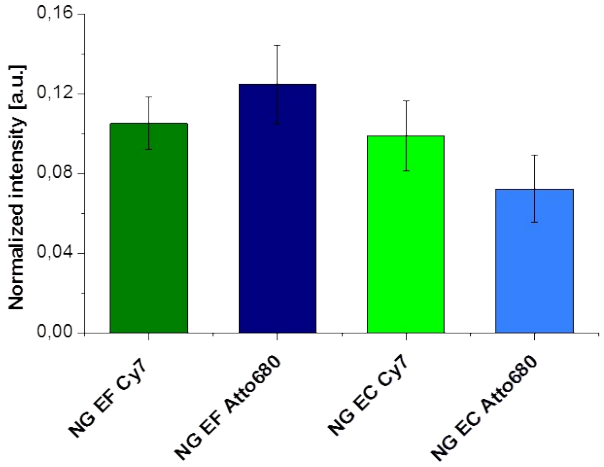


**Figure S 12.** *Ex vivo* tumor fluorescence intensity 72 h after injection showing tumor accumulation of NGs depending on side chain-chemistry and hydrophobic versus hydrophilic character of the probes Cy7 and Atto680. Values were normalized to the fluorescence of the sample before injection.

In case of tumor accumulation tendencies Figure S12 indicates that the attachment of the hydrophilic probe Atto680 enhances the influence of side chain chemistry showing lower accumulation for ester containing then for ester free NGs. In contrast the attachment of Cy7 does not cause this differentiation, probably due to stronger aggregation forces of the hydrophobic probe.

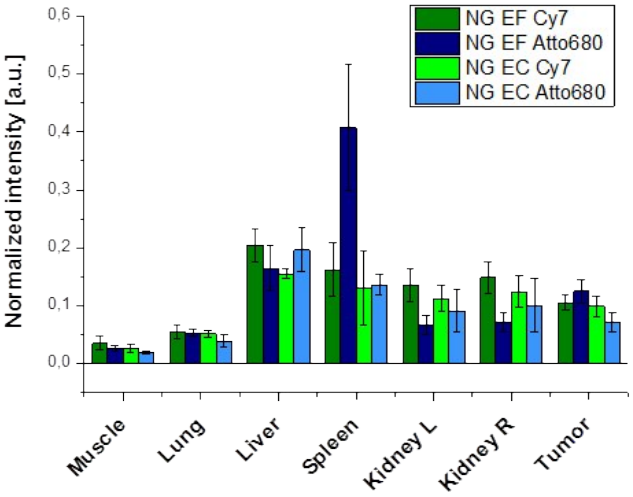


**Figure S 13.** *Ex vivo* organ and tumor fluorescence intensity (normalized by the fluorescence signal of the samples before injection) 72 h after injection of NGs depending on side chain-chemistry and hydrophobic versus hydrophilic character of the probes Cy7 and Atto680.

Figure S13, showing the biodistribution of the NGs in selected organs, does underline the impact of the attached probe especially when comparing the ester free compounds NGEF Cy7 and NG EF Atto680. A strong difference in accumulation is seen in the spleen, where around double the fluorescence intensity of Atto680 is found in comparison to Cy7. In the kidneys the hydrophilic Atto680 does lead to less accumulation.

## References ESI

ESI1) A. O. Fitton, J. Hill, D. E. Jane and R. Millar, *Synthesis*, 1987, **1987**, 1140-1142.

ESI2) L. Hervé, A. Koenig, A. Da Silva, M. Berger, J. Boutet, J. M. Dinten, P. Peltié and P. Rizo, *Applied Optics*, 2007, **46**, 4896-4906.

ESI3) S. Stichler, T. Jungst, M. Schamel, I. Zilkowski, M. Kuhlmann, T. Böck, T. Blunk, J. Teßmar and J. Groll, *Annals of Biomedical Engineering*, 2016, 1-13.