SUPPORTING INFORMATION

Synthesis and Functionalization of Dextran–Based Single–Chain Nanoparticles in Aqueous Media

Raquel Gracia,[a] Marco Marradi,[a] Unai Cossío,[b] Ana Benito,[a] Adrián Pérez-San Vicente,[a] Vanessa Gómez-Vallejo,[b] Hans-Jürgen Grande,[a] Jordi Llop,[b] Iraida Loinaz[a,*]

[a]Biomaterials Unit, IK4-CIDETEC, Pº Miramón 196, 20014, Donostia-San Sebastián, Spain; [b]Radiochemistry and Nuclear Imaging Group, CIC biomaGUNE, Pº Miramón 182, 20014, Donostia-San Sebastián, Spain.

Materials and methods

Dextran from *Leuconostoc* spp. (DXT-40, Mr ~40 kDa), glycidyl methacrylate (GMA) (97%), dimethyl sulfoxide (DMSO) (98%), 3-mercaptopropionic acid (≥99%), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMTMM·HCl) (96%) and 2,2′-(ethylenedioxy)diethanethiol [3,6-dioxa-1,8-octane-dithiol (**DODT**)] (95%) were purchased from Aldrich. Phosphate-buffered saline (PBS) was purchased from Scharlau. 4-(Dimethylamino)pyridine (DMAP) was purchased from Acros-Organics. 2,2'-(7-(4-((2-Aminoethyl)amino)-1-carboxy-4-oxobutyl)-1,4,7-triazonane-1,4-diyl)diaceticacid (**NH_2-NODA-GA**) (98%) was purchased from CheMatech. Water (H_2O) used in the syntheses, unless otherwise stated, was deionized water from a MilliQ A10 Gradient equipment (Millipore).

Dynamic Light Scattering (DLS): DLS analyses were conducted using a Zetasizer Nano ZS, ZEN3600 Model (Malvern Instruments Ltd). All measurements were performed in disposable sizing cuvettes at a laser wavelength of 633 nm and a scattering angle of 173°, while the zeta-potential measurements were performed in disposable zeta potential cells (pH 7.4, 25 ºC). Before the measurement, the samples were dispersed in saline solution (0.9 wt% NaCl for size measurements and 1 mM NaCl for zeta-potential measurements) at a concentration of 1 mg/mL. Each measurement was repeated for three runs per sample at 25 °C.

Gel permeation chromatography (GPC): The weight-average molecular weight (*Mw*), number-average molecular weight (*Mn*) and polydispersity index (PDI; *Mw/Mn*) were measured at 40 ºC on an Agilent GPC-50 system equipped with 2x PL-Aquagel Mixed-

OH, Guard-Aquagel-OH columns and a differential refractive index (RI) detector. 0.3 M $NaNO_3$, 0.01 M NaH_2PO_4, pH 7 was used as eluent at a flow rate of 1 mL/min. The system was calibrated using polyethylene oxide (PEO) standards.

Transmission electron microscopy (TEM): TEM analyses were performed in a TECNAI G2 20 TWIN microscope (FEI, Eindhoven, The Netherlands), operating at an accelerating voltage of 200 KeV in a bright-field image mode. One drop of the sample dispersion in water (~3 μL, 0.035 mg/mL) was deposited on a carbon film supported on a copper grid (300 mesh), hydrophilized by a glow discharge process just prior to use. After staining for 20 seconds with a uranyl acetate aqueous solution (1% w/v), the sample was rotated at high speed in order to dry at room temperature quickly by spinning process. Number-average diameter was calculated by ImageJ platform analysis using a Gaussian curve fitting after counting about 300 nanoparticles.

Nuclear magnetic resonance (^{1}H NMR and DOSY NMR): NMR spectra were recorded on a Bruker AVANCE III spectrometer at 500 MHz and 25 ºC. Chemical shifts (δ) are given in ppm relative to the residual signal of the solvent. Splitting patterns: b, broad; s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet.

Diffusion Coefficient calculations: Taylor dispersion analysis (TDA) studies were performed on a Viscosizer-TD using fused silica capillaries. The mobile phase was water and the solutes were monitored by UV absorbance (UV wavelength filter 214 nm) at two fixed windows. Diffusion coefficient experiments were run at 25 ºC and 140 mbar.

Fourier transform infrared (FTIR) spectra were registered at room temperature in a Jasco FT/IR 4100 spectrophotometer, using a Gladi ATR accessory.

Synthesis of the dextran methacrylated precursor polymer [DXT-MA (DS ~52%)]

Dextran methacrylated polymer (**DXT-MA**) was synthesized following a slightly modified published procedure [1S]. Dextran (DXT-40, 1g, 6.2 mmol) was dissolved in 30 mL of dimethyl sulfoxide (DMSO) under a nitrogen atmosphere, to this solution 200 mg of 4-(*N*,*N*-dimethylamino)pyridine (DMAP, 1.6 mmol) was added. Then, 1 mL of

1S van Dijk-Wolthuis, W. N. E.; Kettenes-van den Bosch, J. J.; van der Kerk-van Hoof, A.; Hennink, W. E. *Macromolecules* **1997**, *30*, 3411−3413.

glycidyl methacrylate (GMA, 7.5 mmol) was incorporated and the mixture was stirred at room temperature during 4 days. The reaction was stopped by adding an equimolar amount of concentrated HCl solution (37% v/v, 1.6 mmol, 0.132 mL) to neutralize DMAP. The modified dextran solution was purified by dialysis against distilled water (MWCO 3500 Da) at room temperature until reaching deionized water conductivity values < 1µS (9 days, refreshing with 4 L of deionized water twice per day). Yield: 75%, DS 52%.^{1}H NMR (500 MHz, D_2O) δ ppm: 6.35-6.10 (m, 1H, methacrylic-CH), 5.92-5.72 (m, 1H, methacrylic-CH), 5.54-4.86 (2.4H, including H-1 and H-2/3 MA-substituted), 4.20-3.33 (10.6H, m, rest of Glc), 1.98 (s, 3H, methacrylic-CH_3).

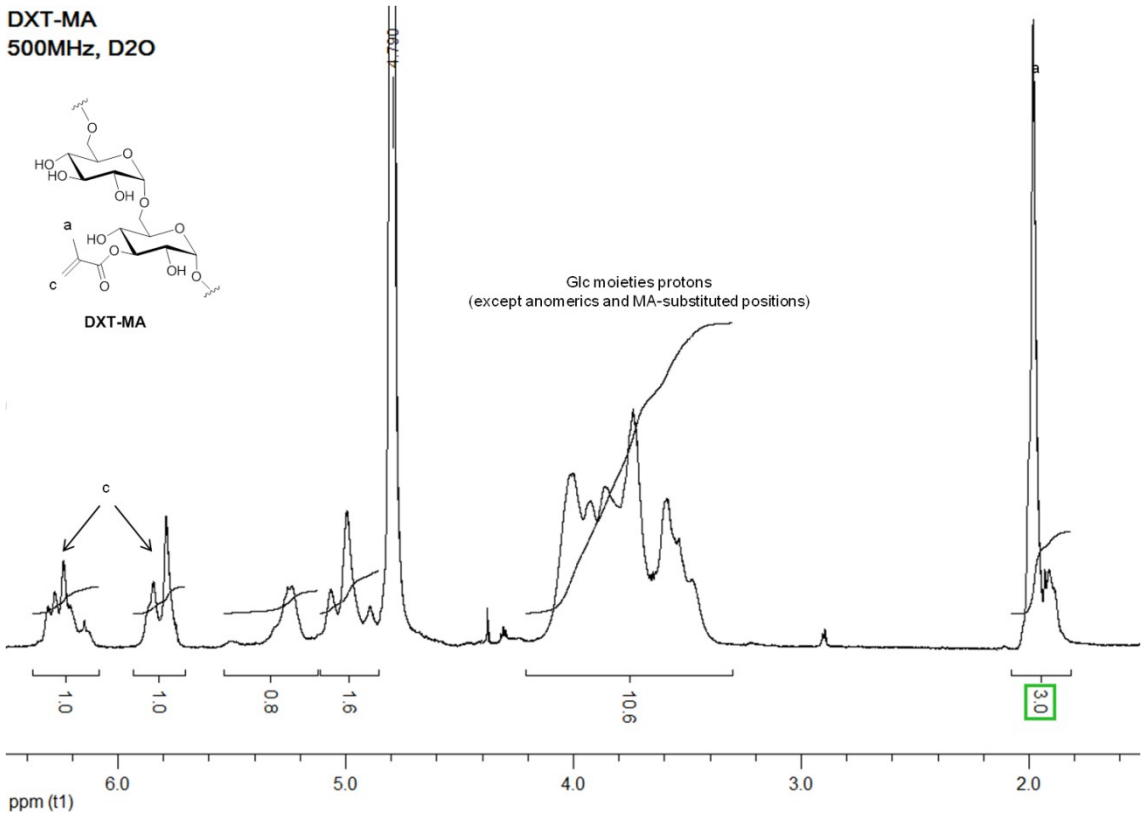

Figure S1: ^{1}H NMR (D_2O, 500 MHz) of **DXT-MA**. For clarity reasons, only MA substitution at position 3 of glucose (Glc) has been depicted. DXT has been drawn as linear polysaccharide (α-1,6 glucosidic linkages) although it is known to be branched, as ramifications (α-1,3 glucosidic linkages) are also present.

The degree of substitution (DS, percent of modified hydroxyl groups per repeating unit) was calculated by ^{1}H NMR through integration of the MA proton signal (integration reference 1.0) with respect to the signals at 3.3-4.2 ppm corresponding to the protons of the glucose (Glc) moiety (6H for unsubstituted Glc and 5H for substituted Glc) except the anomeric protons and the substituted positions (mainly position 3).

Preparation of dextran-based single-chain polymer nanoparticles (DXT-SCPN)

In a standard procedure, 0.38 mL of a previously prepared 0.15 M solution (2 mL, MeOH/PBS, 1:1, v/v, pH= 9.5) of cross-linker **DODT** (0.06 mmol, 49 μL) was added dropwise using a syringe pump (0.05 mL/h) over a 0.02 M solution of **DXT-MA** (DS=52%) (100 mg, 0.025 mmol, 13 mL PBS, pH= 9.5) during 8 h at room temperature and under constant stirring. After addition, the reaction was maintained stirred at room temperature for 12 h. Then, the disappearance of the -SH groups from the homobifunctional cross-linker **DODT** was checked by Ellamn´s test. Further characterization studies were carried out after purification of 5 mL sample from the reaction mixture by dialysis against distilled water (MWCO 3500 Da) until reaching deionized water conductivity values < 1μS (5 days, refreshing with 4 L of deionized water twice per day). Finally the resulting aqueous solution was freeze-dried to obtain nanoparticles as a white solid. Yield >90%. ^{1}H NMR (500 MHz, D_2O) δ ppm: 6.34-6.12 (m, 1H, methacrylic-CH), 5.94-5.70 (m, 1H, methacrylic-CH), 5.55-4.85 (5.5H, including H-1 and H-2/3 MA-substituted), 4.34-3.28 (28H, m, rest of Glc and 2xCH_2O of cross-linker), 3.06-2.53 (5H, m, C*H*(CH_3)C*H*$_2$S, CH_2S of cross-linker), 1.98 (s, 3H, methacrylic-CH_3), 1.29 (s, 3H, cross-linker-CH_3).D_h (DLS) = 13 ± 8 nm; PDI 0.2.

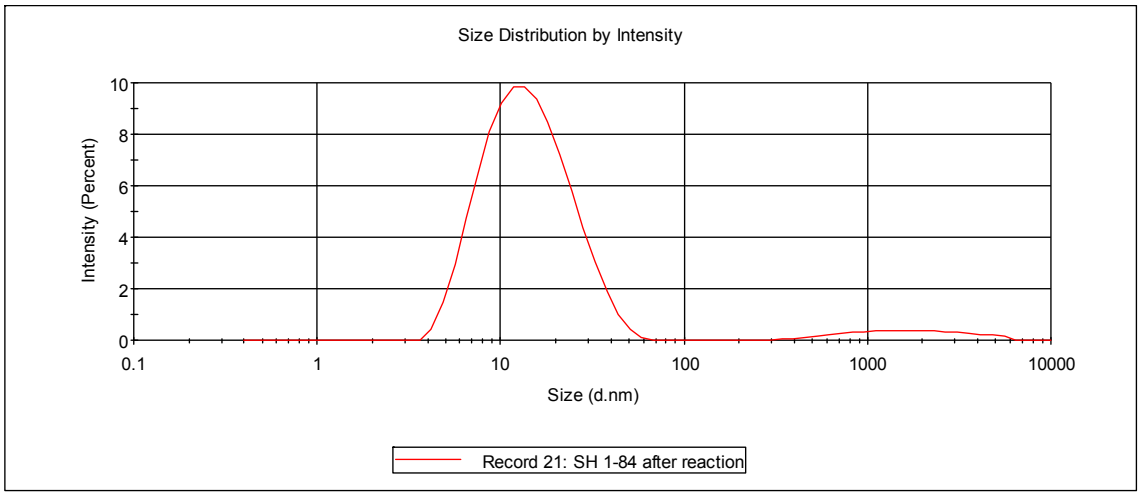

Figure S2: DLS (PBS 10 mM, pH 7.4, 25 ºC) of **DXT-SCPN**.

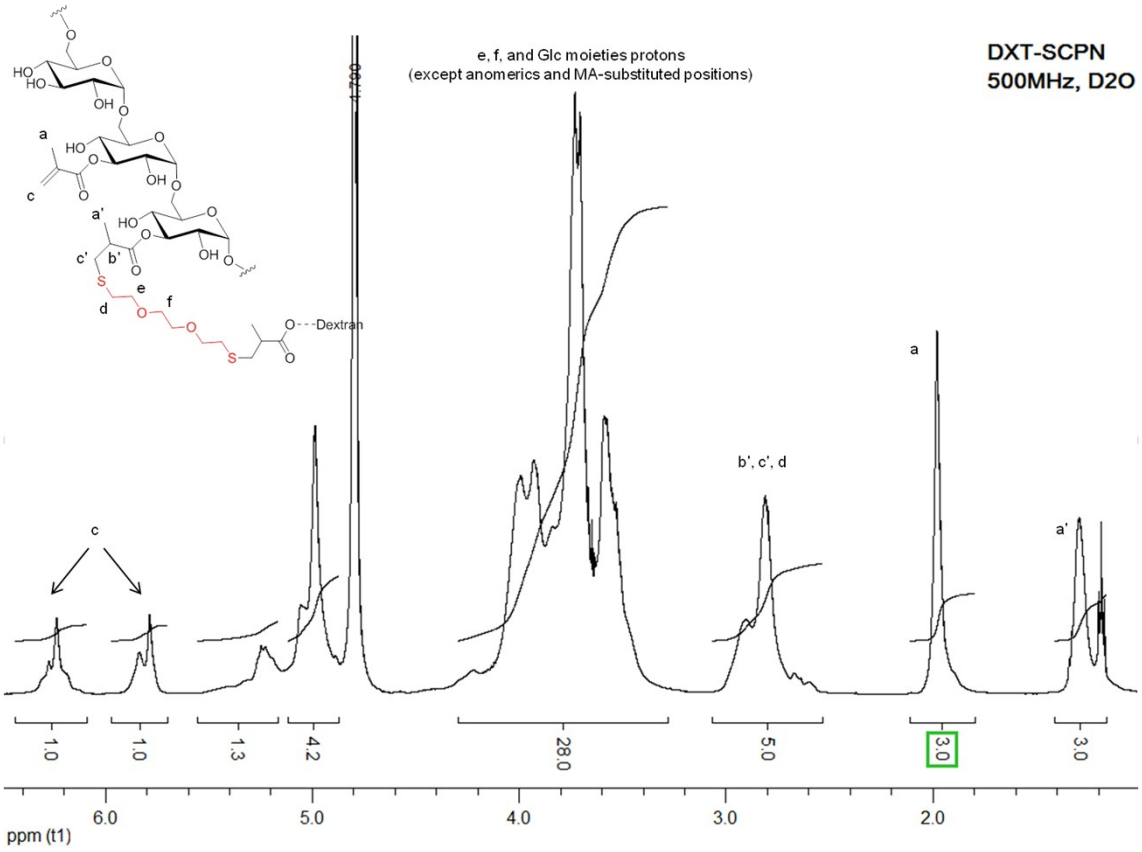

Figure S3: ^{1}H NMR (D_2O, 500 MHz) of **DXT-SCPN**.

Functionalization of DXT-SCPN with 3-mercaptopropionic acid (DXT-SCPN-F)

One batch synthesis of the functionalized **DXT-SCPN-F** was achieved by adding slowly 2 mL of an aqueous solution of 3-mercapto propionic acid (61.4 µL, 7.5 µmol, pH= 9.5) to the reaction flask in the previously reported synthesis of the **DXT-SCPN**. The reaction was stirred for 24 h and the excess acid was removed by dialysis against distilled water (MWCO 3500Da) until reaching deionized water conductivity values < 1µS (5 days, refreshing with 4L of deionized water twice per day). The resulting aqueous solution was freeze-dried to obtain nanoparticles as a white solid. Yield >90%. ^{1}H NMR (500 MHz, D_2O) δ ppm: 5.45-4.90 (6H, including H-1 and H-2/3 MA-substituted), 4.13-3.41 (31H, m, rest of Glc and 2xCH_2O of cross-linker), 3.02-2.71 (8H, m, 2x C*H*(CH_3)C*H*$_2$S, CH_2S of cross-linker and MPA), 2.70-2.49 (2H, m, C*H*$_2$COOH of MPA), 1.29 (s, 5.4H, cross-linker- and MPA-CH_3) *Mw* (GPC) = 38KDa, *Mw*/*Mn* = 1.7; D_h (DLS) = 15 ± 4 nm; PDI 0.2, $Zeta_{potential}$ (pH = 7.2, 25 °C) = -20mV ± 5. TEM (uranyl acetate staining): 13 ± 3 nm.

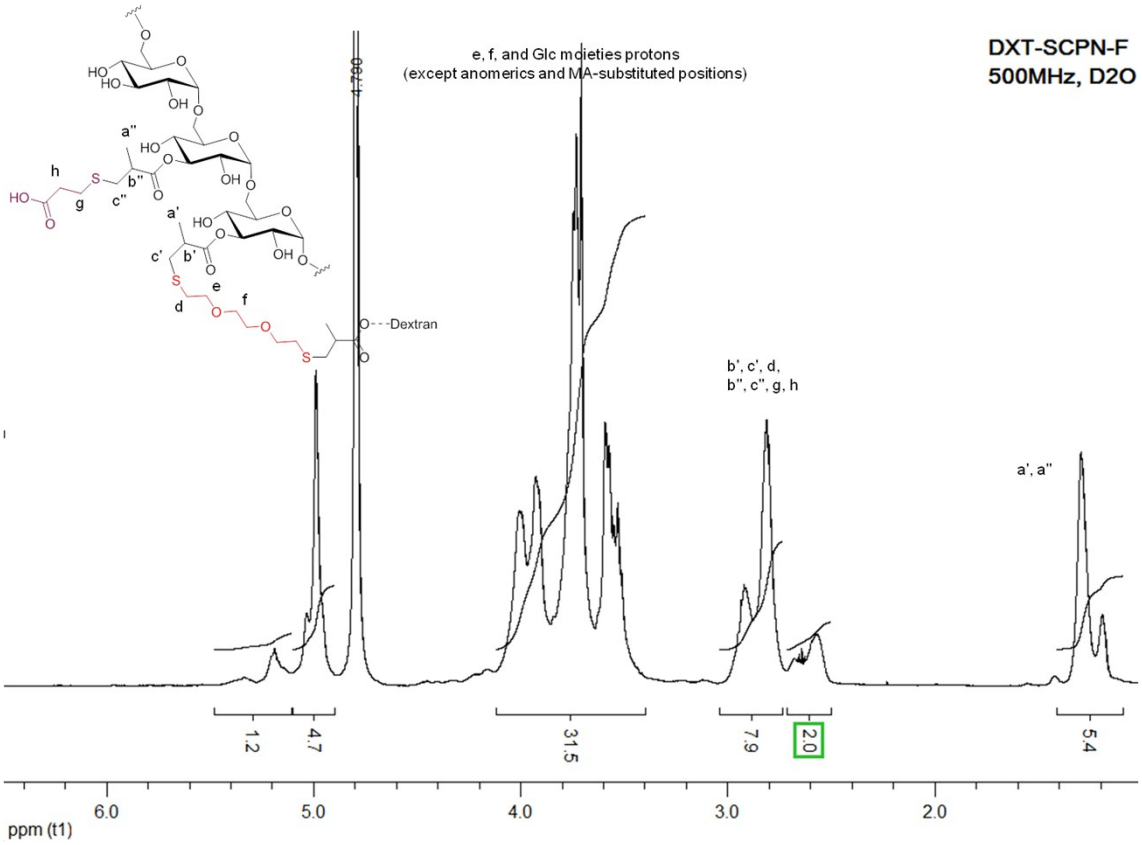

Figure S4: ^{1}H NMR (D_2O, 500 MHz) of SCPNs functionalized with MPA (**DXT-SCPN-F**).

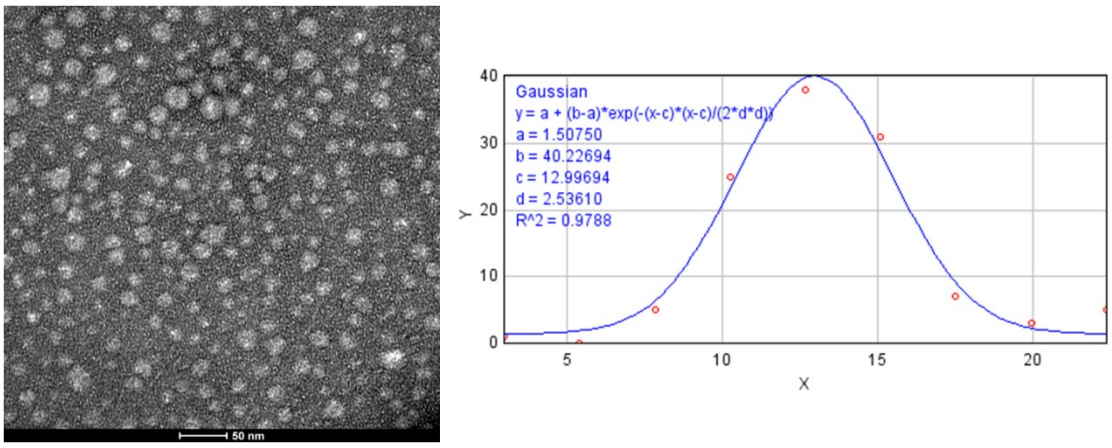

Figure S5: TEM (uranyl staining) and distribution analysis (number-average diameter calculated by ImageJ platform using a Gaussian curve fitting after counting about 300 nanoparticles) of **DXT-SCPN-F**.

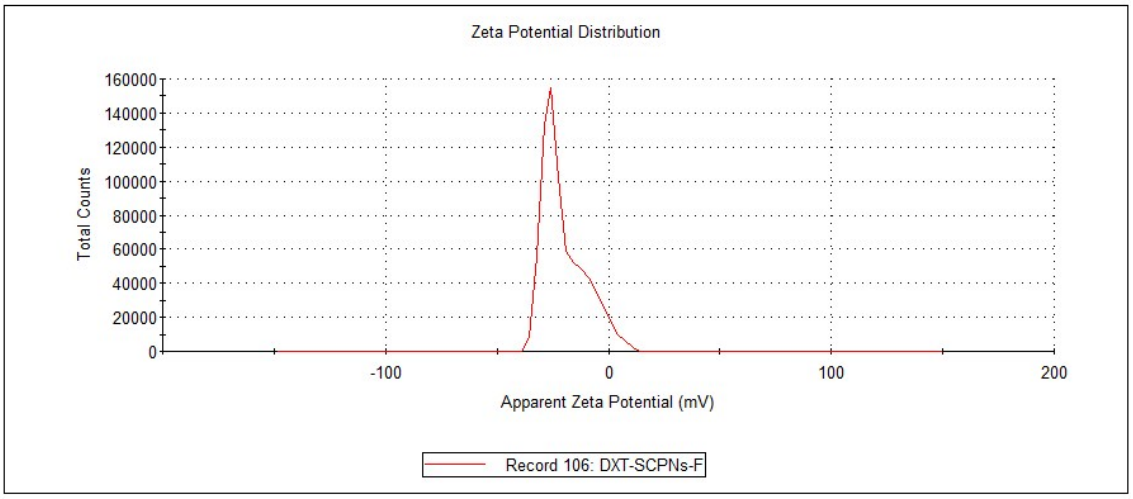

Figure S6: Z-potential measurement (1mM NaCl) of **DXT-SCPN-F** (pH = 7.2, 25 °C).

Functionalization of the DXT-MA (52%) with 3-mercaptopropionic acid (DXT-MA-F)

An aqueous solution of 3-mercaptopropionic acid (430 µL, 5 mL H_2O, pH= 9.5) was slowly added to a previously prepared solution of **DXT-MA** (DS 52%) (350 mg, 20 mL H_2O, pH= 9.5). The reaction was maintained under constant stirring for 12 h and then purified by dialysis against distilled water (MWCO 3500Da). The resulting aqueous solution was freeze-dried to obtain the resulting quenched polymer as a whitesolid. *Mw* (GPC) = 47 KDa, *Mw*/*Mn* = 1.7. $Zeta_{potential}$ (pH = 7.2) = -12 mV ± 7.

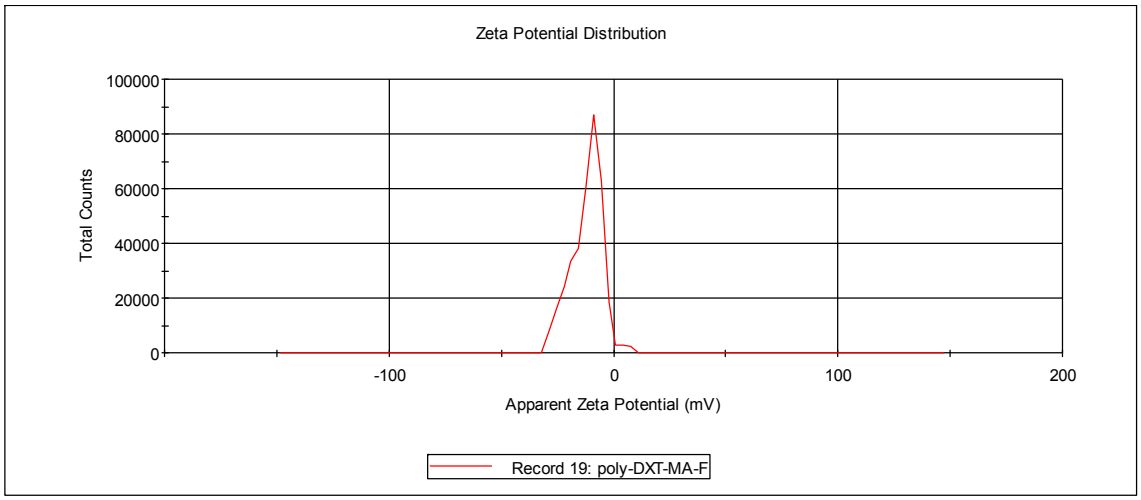

Figure S7: Zeta potential measurement (1 mM NaCl) for **DXT-MA-F** (pH = 7.2, 25°C).

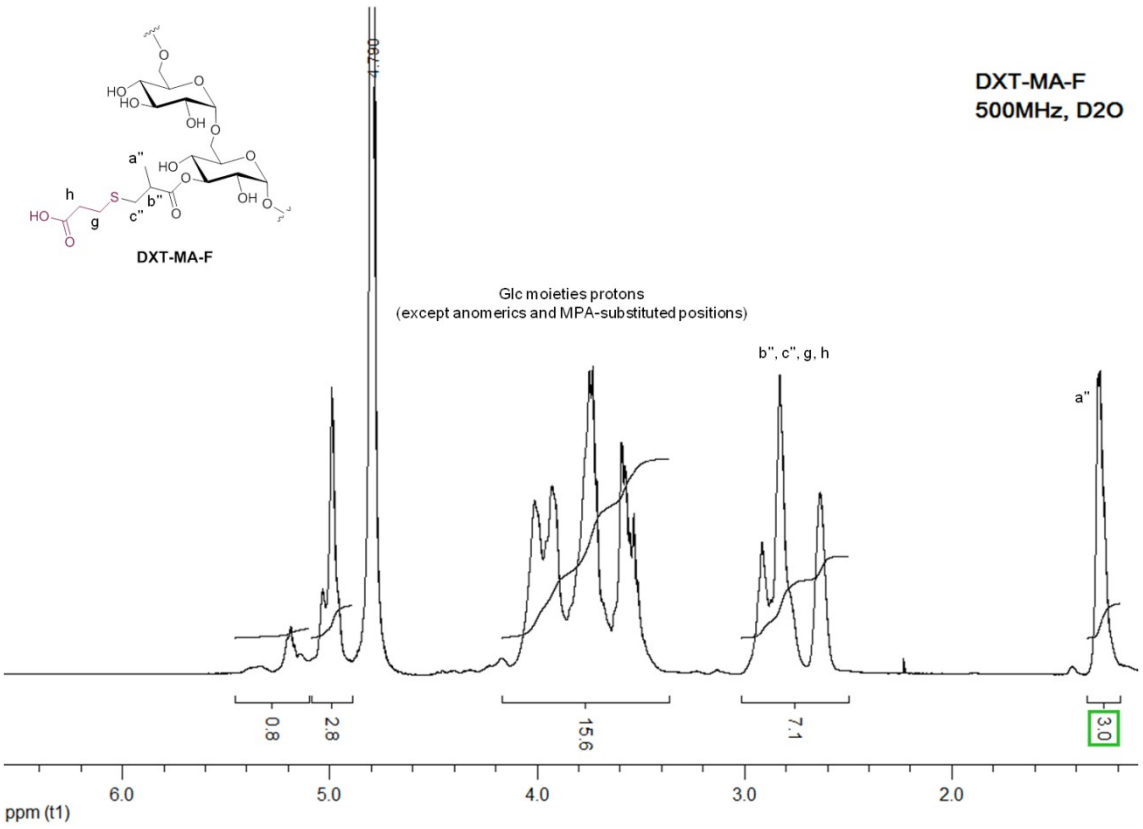

Figure S8: ^{1}H NMR (D_2O, 500 MHz) of **DXT-MA** functionalized with mercaptopropionic acid **(DXT-MA-F)**.

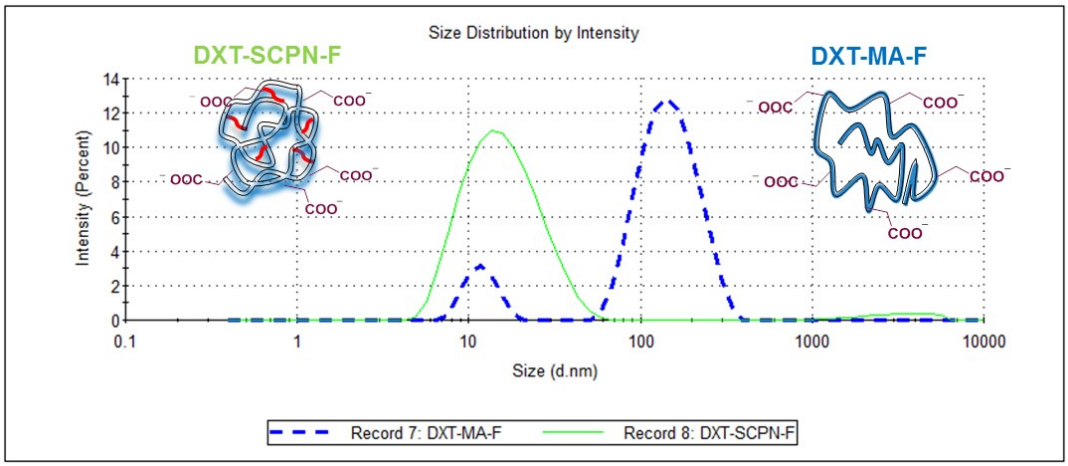

Figure S9: DLS (PBS 10 mM, pH 7.4, 25 ºC) of DXT-SCPN-F (green trace) and DXT-MA-F (dashed blue trace).

Comparison of FT-IR spectra

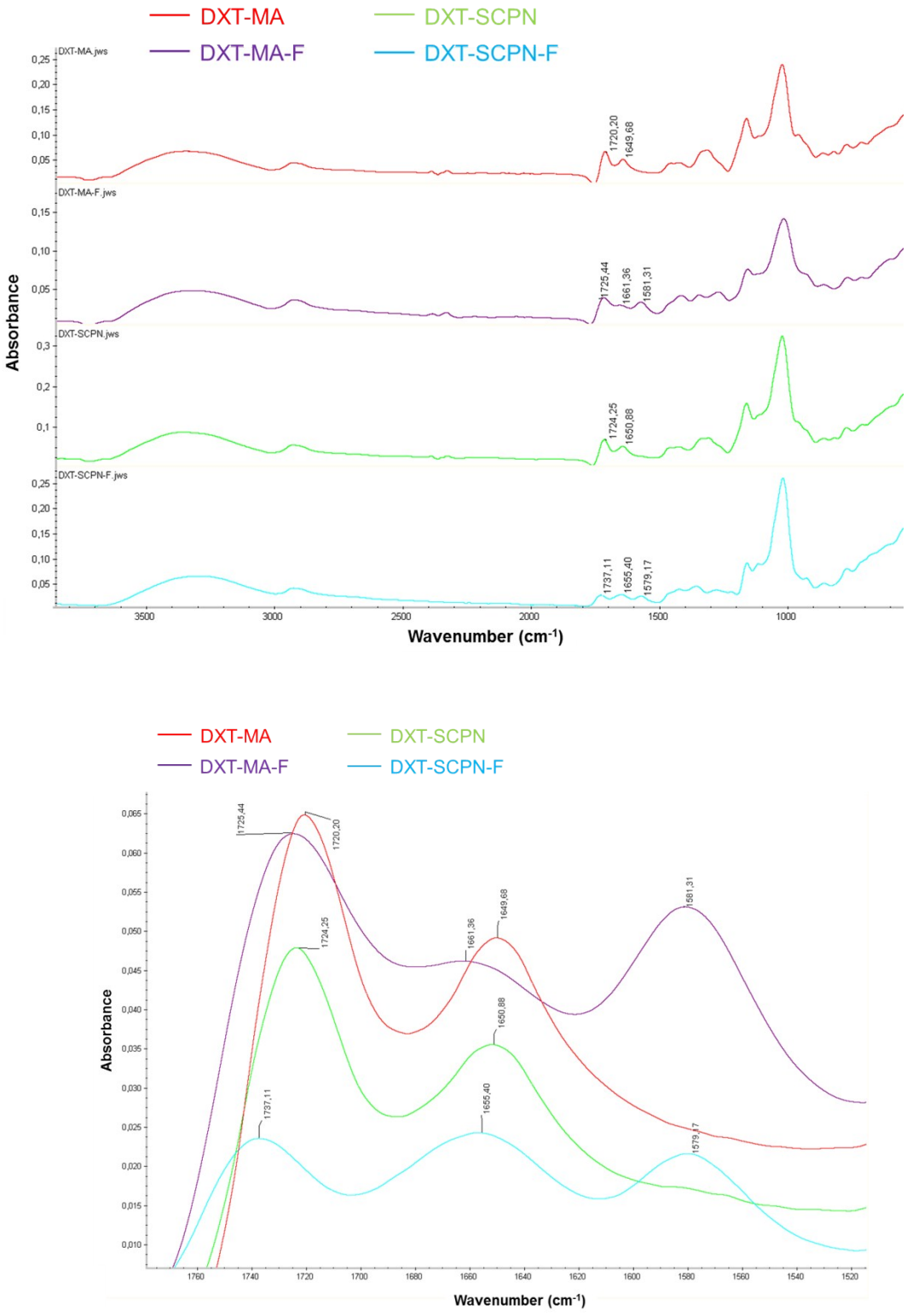

Figure S10: Full FT-IR (up) and insight (bottom) of dextran methacrylated precursor polymer (**DXT-MA**, in red), the corresponding single-chain nanoparticles (**DXT-SCPN**, in green), the MPA-functionalized DXT-SCPN (**DXT-SCPN-F**, in blue) and the polymer control system which consists of MPA-functionalized DXT-MA (**DXT-MA-F**, in violet).

Cytotoxicity

In vitro cytotoxicity studies were carried out with the MTS assay, after 48 h of exposure to increasing concentrations of nanoparticle ranging from 6.25 to 50 µg/mL.

Cell viability analysis showed that under the conditions of this study none of the tested concentrations caused a significant decrease in cell viability when compared with none treated cells (Figure S7). Moreover, morphological analysis of Hela cells showed a typical epithelial adherent morphology.

Cell culture: HeLa cells were cultured in medium containing EMEM (Gibco), 10% fetal bovine serum (Lonza), 1X non-essential amino acid (NEAA) (Sigma), 1% penicillin/ streptomycin (Sigma), 2 mM L glutamine (Sigma). Trypsin (0.25%)/EDTA were used to harvest the cells at 80% confluence. Cell cultures were maintained at 37 ºC in an incubator with 95% humidity and 5% of CO_2. The cultures were replenished with fresh medium at 37 ºC twice a week.

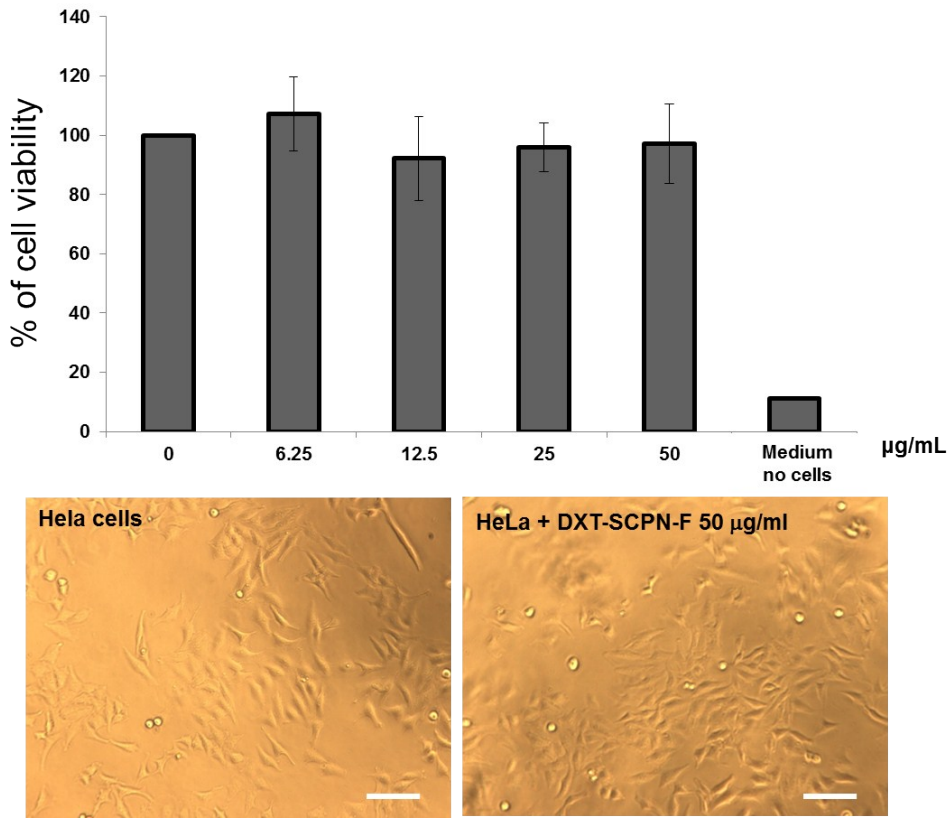

Figure S11: Cytotoxicity studies. MTS cell viability assay of HeLa cell exposed to increasing concentrations of dextran-based single-chain nanoparticles (**DXT-SCPN-F**) ranging from 6.25 to 50 µg/mL. Scale bar 200 µm.

MTS assay: HeLa cell growth was evaluated using Cell Titer 96 ® Aqueous One Solution Cell proliferation Assay (Promega). HeLa cells were seeded at a density of 10000cl/cm^2 in p96 well plates and allowed to grow for 24 h. After removing the medium, 100 µl of HeLa medium containing various concentrations nanoparticles ranging from 6.25 to 50 µg/mL were added and further incubated for 48 h. At 48 h post nanoparticles incubation, cells were cultured in a 37 ºC of humidified incubator for 2 h with 20µL of Cell Titer 96 ® Aqueous One Solution Reagent containing tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4 sulfophenyl)-2H-tetrazolium)] and an electron coupling reagent, phenazine ethosulfate (PES) per 100 µL of cultured media. The absorbance per well was measured at 490 nm using a micro-plate reader (Multiscan ascent, Thermo). All experiments were performed in triplicate.

Functionalization of the SCPNs with NODA and radiolabeling for *in vivo* biodistribution studies

Functionalization: To a stirred solution of **DXT-SCPN-F** (20 mg) in DMSO (8 mL), 10mg of DMTMM·HCl (0.04 mmol) were added and the mixture was maintained under constant stirring for 15 min. Then, a solution of **NH_2-NODA-GA** (10mg, 0.02mmol) in DMSO (1mL) was added to the reaction flask and the reaction was stirred at room temperature for 12 h. The reaction was purified by dialysis against distilled water (MWCO 1000 Da) until reaching deionized water conductivity values (< 1 µS). The amount of NODA (0.1mg NODA/1mg **DXT-SCPN-F**) was calculated by loading Zn(II) which concentration was determined by colorimetric titration in acetate buffer (pH=4.5) using xylenol orange as the indicator. D_h (DLS) = 14 ± 9 nm; PDI 0.2, $Zeta_{potential}$ (pH =7.2) = -12 mV ± 7.

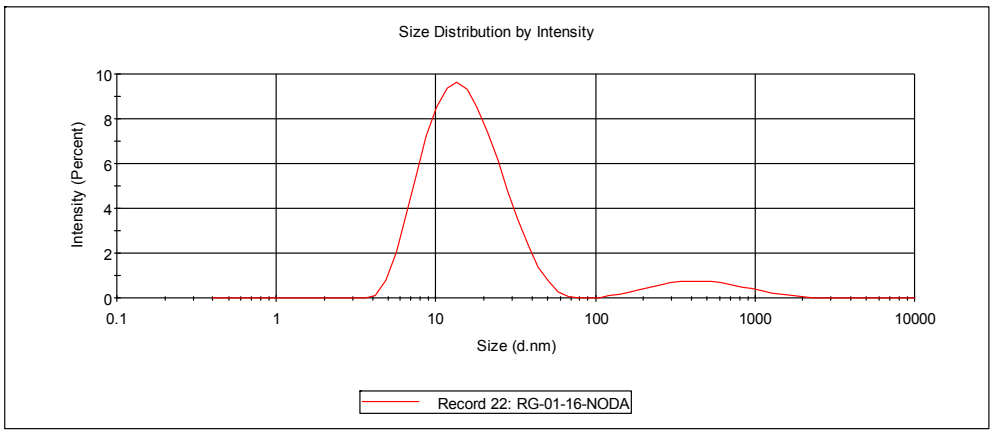

Figure S12: DLS (PBS 10 mM, pH 7.4, 25 ºC) of **DXT-SCPN-NODA**.

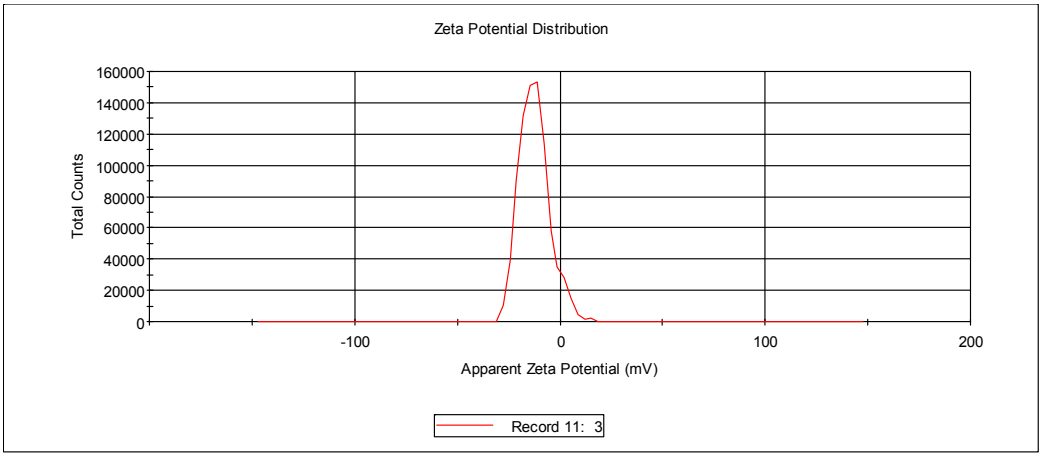

Figure S13: Zeta potential measurement (1mM NaCl) for NODA-functionalized SCPNs (**DXT-SCPN-NODA**) (pH = 7.2, 25°C).

Radiolabelling of SCPNs: Gallium-67 (^{67}Ga) was obtained from Mallinckrodt Medical, B.V. (Le Petten, The Nederlands) as citrate salt. Prior to radiolabelling reactions, ^{67}Ga citrate was converted into ^{67}Ga chloride using a previously described method, with minor modifications [2S]. In brief, the ^{67}Ga citrate solution was first eluted through two silica cartridges connected in series (Sep-Pak® Silica Plus Light, Waters Co., Milford, MA, USA) at a constant flow rate of 0.1 mL/min. The cartridges were dried with air for 1 minute and washed with ultrapure water (5 mL, obtained from a Milli-Q® Purification System, Millipore®, Merck KGaA, Darmstadt, Germany). Desorption of ^{67}Ga ions was finally achieved by elution with 0.1 M aqueous HCl solution (1 mL) at a flow rate of 0.1 mL/min. The eluate was collected in different fractions (ca. 100 µL each) and those fractions containing the maximum activity concentration (typically fractions 4-5) were used in subsequent labelling experiments.

Radiolabeling of SCPNs was performed by incubation of the NODA-functionalized nanoparticles (**DXT-SCPN-NODA**) with $^{67}GaCl_3$. In a typical experiment, 50 µL of SCPNs solution (1 mg/mL) and 50 µL of $^{67}GaCl_3$ were incubated in sodium acetate buffer solution (0.2M, pH = 4.2, 200 µL). The mixture was incubated at 25°C for 45 min. After incubation, the crude material was purified by centrifugal filtration using Millipore Amicon® Ultra filters (3 kDa cut-off). The resulting precipitate was washed three times with sodium acetate buffered solution to remove unreacted ^{67}Ga species, and the amount of radioactivity in the pellet, the supernatant and the washings were determined in a dose calibrator (CPCRC-25R, Capintec Inc., NJ, USA). Labelling efficiency (expressed in

2S Chan, K.; Gonda, I. *Eur. J. Nucl. Med*. **1991**, *18*, 860 (A simple method for the preparation of gallium chloride from the citrate solution).

percentage) was calculated as the ratio between the amount of radioactivity in the filter and the total amount of radioactivity in all fractions. Finally, the nanoparticles were suspended in 0.2 M sodium acetate buffer solution (pH 4.2). Radiochemical yield was calculated as the ratio between the amount of radioactivity in the resuspended fraction and the starting amount of radioactivity.

Radiochemical stability of SCPNs: Radiolabelled nanoparticles prepared as described above were incubated in sodium acetate buffered solution at 37 °C using a digital block heater. At different time points (1, 3, 24, 48, 72 and 144 h) samples were withdrawn and the amount of radioactivity was measured. The ^{67}Ga-radiolabelled SCPNs were filtered, washed twice with ultrapure water, and the amount of radioactivity in the filter and the filtrate/washings was measured. The radiochemical stability was calculated as the percentage of radioactivity in the pellet with respect to the total amount of radioactivity (pellet + filtrate + washings).

Animal studies: Animals were maintained and handled in accordance with the Guidelines for Accommodation and Care of Animals (European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes). All animal procedures were performed in accordance with the Spanish policy for animal protection (RD53/2013), which meets the requirements of the European Union directive 2010/63/UE regarding their protection during experimental procedures. Experimental procedures were approved by the Ethical Committee of CIC biomaGUNE and authorized by the regional government.

Administration of the radiolabelled SCPNs: Six-to–eight weeks-old female Sprague Dawley rats (Janvier, Le Genest-Saint-Isle, France) were used. Rats were anesthetized by an intraperitoneal injection of a mixture of medetomidine, midazolam and fentanyl (0.6, 6 and 0.02 mg/Kg, respectively). Once animals (n = 4) were under sedation, ^{67}Ga-radiolabelled SCPNs were administered by intratracheal nebulization using a Penn-Century MicroSprayer® Aerosolizer (FMJ-250 High Pressure Syringe Model, Penn-Century. Inc. Wyndmoor, USA). A small animal Laryngoscope (Model LS-2, Penn-Century. Inc.) was used for correct visualization of the epiglottis, ensuring a correct positioning of the tip just above the carina. A pre-defined volume of radiolabelled SCPNs (50 µL, established by using spacers in the syringe plunger) was administered (amount of

radioactivity around 1.85 MBq). Immediately after, rats were submitted to *in vivo* imaging studies.

In vivo imaging studies: Immediately after administration of the radiolabeled SCPNs, and without recovering from sedation, animals were positioned in an eXplore speCZT CT preclinical imaging system (GE Healthcare, USA) to perform *in vivo* studies. Body temperature was maintained with a homeothermic blanket control unit (Bruker BioSpin GmbH, Karlsruhe, Germany) to prevent hypothermia, and SPECT scans were acquired for 30 min. After the SPECT scan, a CT acquisition was performed to provide anatomical information of each animal. The SPECT images were reconstructed using a ordered-subset expectation maximization (OSEM) iterative algorithm (3 iterations/3 subsets, 128 x 128 x 32 array with a voxel size of 0.55 x 0.55 x 2.46 mm^3), whereas for the CT a cone beam filtered back-projection a Feldkamp algorithm (437 x 437 x 800 array with a voxel size of 0.2 x 0.2 x 0.2 mm^3) was used. After reconstruction, images were quantified using πMOD analysis software (version 3.4, PMOD Technologies Ltd.). Volumes of interest (VOIs) were manually drawn in the lungs on the CT images and translated to the SPECT images. The relative concentration of radioactivity in the different VOIs was finally determined.