Supporting Information for

Tetrazine – *trans*-cyclooctene chemistry applied to fabricate self-assembled fluorescent and radioactive nanoparticles for *in vivo* dual mode imaging

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1. Materials and instrumentation

Unless stated otherwise, all reagents and chemicals were obtained from commercial sources and used without further purification. Water was purified on an EMD Millipore Milli-Q integral water purification system. Ghosez reagent, 2-bromofluorene, tetrakis(triphenylphosphine)palladium(0), 4 M HCl in dioxane, triethylamine and DIPEA were bought from Sigma-Aldrich. NHS-TCO was in detail characterized- and provided by SyMO-Chem / Synthon B.V. Tetrazine-PEG_{11}-DOTA (tz-DOTA) was synthesized and characterized by SyMO-Chem and provided by Tagworks Pharmaceuticals. Indium-111 chloride was purchased from PerkinElmer. Labeling buffers were treated with Chelex-1000 (BioRad Laboratories) overnight and filtered. Instant-thin layer chromatography plates (iTLC) were bought from Varian Inc. Zeba spin desalt columns were bought from Fisher. NHS-DOTA were in detail characterized- and provided by SyMO-Chem. All solvents were of AR or HPLC quality and purchased from Biosolve. Deuterated chloroform was dried over 4 Å molsieves and deuterated acetone was used from capsules. Water was purified on an EMD Millipore Milli-Q integral water purification system. Thin-layer chromatography was performed with 0.25 mm 60F-254 precoated silica plates or 60 RP-18 F254S plates from Merck.

All the NMR data were recorded on a Varian Mercury Vx 400 MHz NMR for ^{1}H-NMR (100 MHz for ^{13}C-NMR). Proton experiments are reported in parts per million (ppm) downfield of TMS and were relative to the residual chloroform (7.26 ppm). All ^{13}C spectra were reported in ppm relative to residual chloroform (77 ppm). Splitting patterns are labeled as s, singlet; d, doublet; ddd doublet of doublet of doublets; dt, double triplet; dd, double doublet; t, triplet; q, quartet; m, multiplet. Silica column chromatography was performed using silica with particle size 60 – 200 µm. Matrix-assisted laser desorption/ionisation-Time of Flight mass spectrometry was performed on a PerSeptive Biosystems Voyager DE-PRO spectrometer using α-cyano-4-hydroxycinnamic acid (CHCA) and 2-[(2E)-3-(4-tert-butylphenyl)-2-methylprop-2-enylidene]malononitrile (DCTB) as matrices. UV-spectra were recorded on a Perkin Elmer Lambda950 or Perkin Elmer Lambda 900. Dynamic light Scattering (DLS) measurements were performed on a Malvern Zetasizer ZMV2000. Fluorescent spectroscopy was performed on a Varian Cary Eclipse fluorescence spectrometer with temperature controlled multi-cell holder. TLC strips were evaluated with a phosphor imager (FLA-7000, Fujifilm) with AIDA software from Raytest). Gamma-counting was performed on a Wizard 1480 from PerkinElmer.

2. Methods

SMNP preparation was performed according to previously published protocol[1]. Briefly solid oligomer was dissolved in tetrahydrofuran (THF) at 1 mM and typically 15 µL or 30 µL of this concentrated solution was injected in 5 mL water or PBS, resulting in a SMNP solution at respectively 3 µM or 6 µM. Pre-mixing was performed by mixing in desired molar ratio the concentrated THF stocks prior to injection in water. SMNPs were concentrated by careful rotary evaporation with the heating bath at 35°C, increasing the concentration of SMNPs from 6 µM to 30 µM.

Labeling of BCN-DOTA was typically performed by combining 25 µL probe (1.3 mg/mL) with buffer and the desired amount of indium-111 in a total volume of 100 µL. For the reaction with tetrazine-albumin, tetrazine was present in large excess compared to the probe.

Labeling of tz-DOTA was typically performed by combining 2 µL of tz-DOTA (1.46 mM) with 20 µL 0.2 M NH_4OAc buffer and the desired amount of indium-111. The solution was incubated for 5 min at 60°C and DTPA challenged by addition of an excess of DTPA (5 µL of 10 mM DTPA solution) followed by incubation at 60°C for 5 min.

The SMNP labeling studies were typically performed by combining 100 µL of 25 % TCO-SMNPs (6 µM or 30 µM) with 0.1 equivalents of labeled tz-DOTA. The solution was incubated in dark for 30 min at room temperature.

3. Animal experiments

All animal experiments were performed according to the principles of laboratory animal care (NIH publication 85-23, revised 1985) and the Dutch national law "Wet op de Dierproeven" (Stb 1985, 336). The in vivo experiments were performed in tumour-free nude female Balb/C mice (20–25 g body weight, Charles River Laboratories). Each mouse received 100 µL containing 30 µM ^{111}In-labeled SMNPs. Blood sampling was performed at t = 2, 5, 10, 20, 30 and 90 min via the vena saphena. At the end of each experiment, the mice were anesthetized and euthanized by cervical dislocation. Blood was withdrawn by heart puncture, and selected organs and tissues were harvested and blotted dry. All samples were weighed and then combined with 1 mL of water or 4 % formaldehyde / PBS solution for fixation. The sample radioactivity was counted in a gamma counter along with ten standards to determine the percentage injected dose per gram (%ID/g) and the percentage injected dose per organ (%ID/organ) and raw data is presented in tables below.

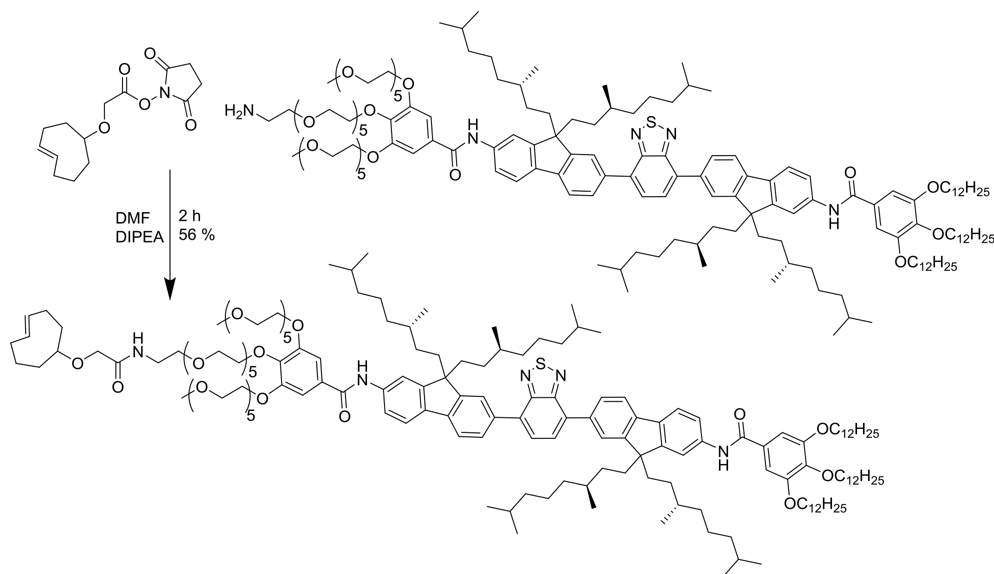
Table S1: Percentage injected dose per gram (% ID/g) or percentage injected dose per organ of ^{111}In- labeled SMNPs in various tissues and on various time points of A) a biodistribution study (data are the mean ± SEM, n = 3) and B) of in a blood clearance experiment (data are the mean ± SEM, n = 4).

A) Biodistribution							
Tissue	**4h**	**SEM**	**24h**	**SEM**	**70h**	**SEM**	
blood	9.03E-02	9.95E-03	8.89E-03	1.78E-03	7.33E-03	1.48E-03	**% ID/g**
heart	3.29E-02	4.22E-03	4.73E-02	1.52E-02	8.04E-03	1.49E-03	
lung	1.67E-01	1.53E-02	1.67E-01	1.90E-02	8.73E-02	7.40E-03	
liver	8.04E+01	3.71E+00	6.87E+01	7.23E-01	6.36E+01	3.25E+00	
spleen	3.48E+01	1.81E+00	2.94E+01	4.65E+00	2.34E+01	4.60E+00	
pancreas	7.02E-02	2.89E-02	1.08E-01	1.65E-02	4.57E-02	2.18E-02	
kidney R	5.74E-01	2.43E-01	4.24E-01	2.48E-02	1.79E-01	1.17E-02	
kidney L	5.05E-01	2.12E-01	4.46E-01	2.49E-02	1.63E-01	8.05E-03	
surrenal glands	1.55E+00	3.89E-01	1.28E+00	7.30E-02	7.35E-01	4.62E-01	
muscle	4.57E-03	4.21E-04	5.35E-02	4.70E-03	1.24E-02	4.51E-03	
bone	1.78E-01	2.23E-02	2.09E-01	1.49E-02	1.47E-01	1.84E-02	
brain	2.36E-03	2.20E-04	7.48E-03	6.57E-03	2.75E-04	4.86E-04	
bladder	2.73E-02	6.99E-03	3.13E-01	2.77E-01	3.61E-02	2.01E-02	
urine	8.81E-01	4.12E-01	9.23E-01	1.16E-01	1.38E+00	3.82E-01	
small intestine	8.03E-02	1.00E-02	1.59E-01	1.62E-02	2.90E-02	1.68E-02	**% ID/organ**
large intestine	6.19E-02	9.43E-03	1.42E-01	4.34E-02	2.97E-03	1.71E-03	
stomach	1.82E-02	3.22E-03	4.39E-02	1.70E-02	3.31E-02	1.91E-02	
tail	5.18E-01	2.08E-01	6.34E-01	4.04E-01	7.89E-02	4.56E-02	

B) Blood clearance		
Time (min)	**% ID/g**	**SEM**
2	1.73E+01	1.32E+00
5	4.40E+00	8.86E-01
10	9.45E-01	2.01E-01
20	2.18E-01	1.43E-02
30	1.39E-01	1.26E-02
90	7.57E-02	5.69E-03

4. Synthetic procedures

<u>Amp</u>-TCO



Scheme S1: Synthesis of **amp**-TCO.

15.1 mg (5.81 µmol, 1 eq) amp1-NH_2[2] was dissolved in 1 mL dry DMF with 6 µL (4.5 eq) DIPEA under inert and dark conditions and 4.33 mg (15.4 µmol, 2.65 eq) (E)-cyclooct-4-en-1-yl 2,5-dioxopyrrolidine-1-carboxylate (NHS-TCO, SyMO-Chem) was added. After 2 hours incubation, crude solution was extracted with $CHCl_3$ / water and washed 2 times and organic phases were concentrated *in vacuo*. Automated column chromatography (SiO_2) was performed using 40 → 95 % THF / heptane and separation was monitored by ELSD detection. Product was isolated in yield of 56 % (9 mg, 3.3 µmol) analysed by MALDI-ToF and NMR. ^{1}H-NMR ($CDCl_3$): δ 8.50 (br, 1H), 8.03 (d, J = 6.9 Hz, 2H), 7.93 (s, 2H), 7.88 – 7.78 (m, 6H), 7.78 – 7.73 (m, 2H), 7.63 (d, J = 7.9 Hz, 1H), 7.55 (d, J = 7.5 Hz, 1H), 7.30 (s, 2H), 7.10 (s, 2H), 5.53 (m, 1H), 4.30 – 4.21 (m, 6H), 4.09 – 4.00 (m, 6H), 3.94 – 3.78 (m, 9H), 3.75 – 3.50 (m, 52H), 3.35 (s, 6H), 2.75 (s, 2H), 2.37 – 2.21 (m, 2H), 2.17 – 1.96 (m, 10H), 1.89 – 1.70 (m, 10H), 1.61 – 1.44 (m, 22H), 1.41 – 0.97 (m, 84H), 0.88 (t, J = 6.9 Hz, 12 H), 0.78 – 0.59 (m, 46H). MALDI-ToF MS: calc. [**M**+**H**]$^+$: 2761.93, found: 2761.96; [**M**+**Na**]$^+$: 2784.92, found: 2784.92; [**M**+**K**]$^+$: 2800.89, found: 2800.92. R_f = 0.45 on Si-TCL using 70 % THF / heptane.

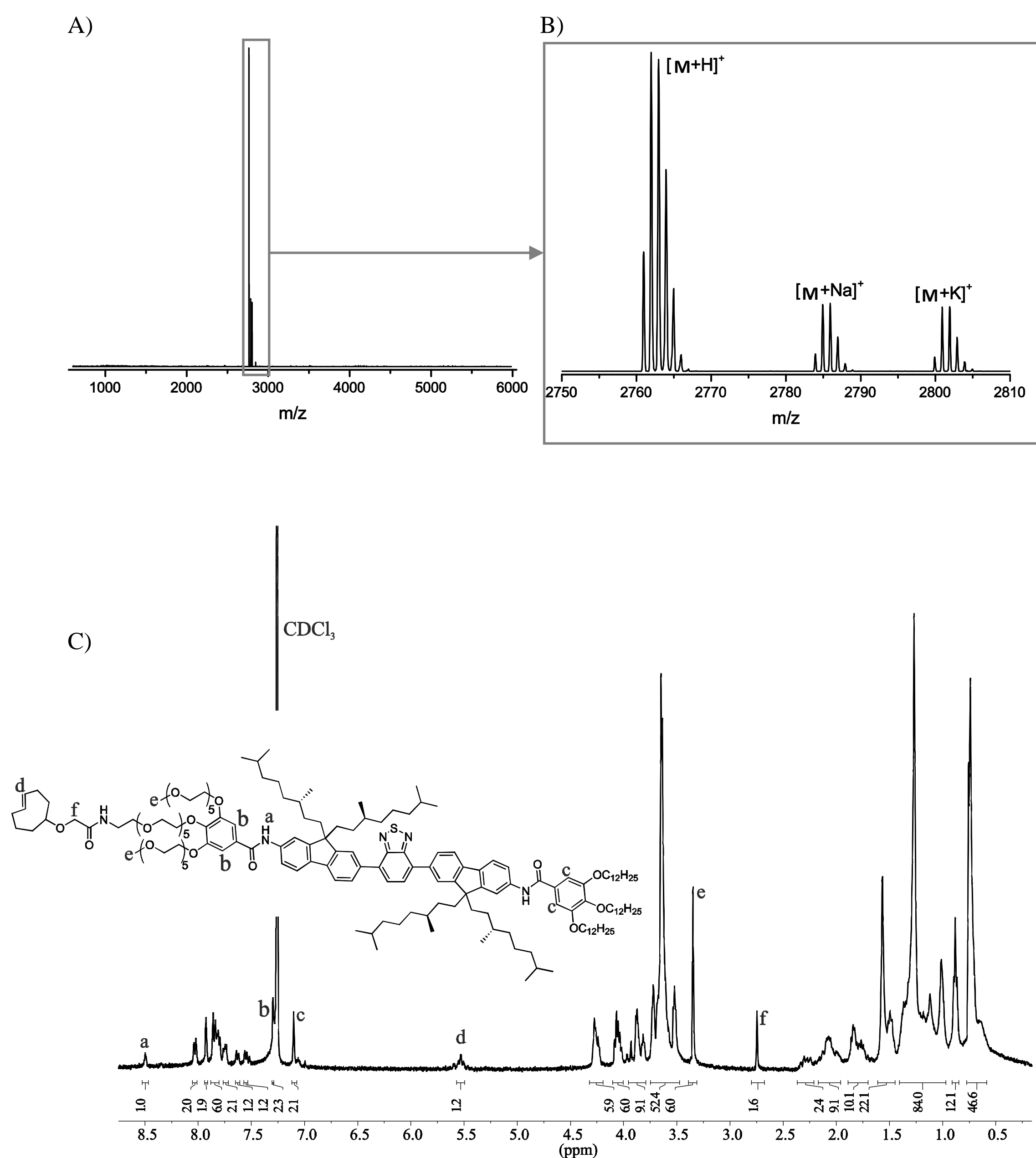


Fig. S1: A) and B) MALDI-ToF spectrum of **amp**-TCO, C) ^{1}H-NMR spectrum of **amp**-TCO in $CDCl_3$.

Amp-TCO - tz-DOTA complex

Unlabeled 0.15 mM tetrazine-DOTA dissolved in water and 1mM **amp**-TCO dissolved in THF were combined (1 : 1, mol) and gently shaken for 1 h and subsequently submitted for MALDI-ToF MS analysis. Two MALDI-ToF matrices were used, namely α-cyano-4-hydroxycinnamic acid (CHCA) and 2-[(2E)-3-(4-tert-Butylphenyl)-2-methylprop-2-enylidene]malononitrile (DCTB) and in both matrices, product formation was observed.

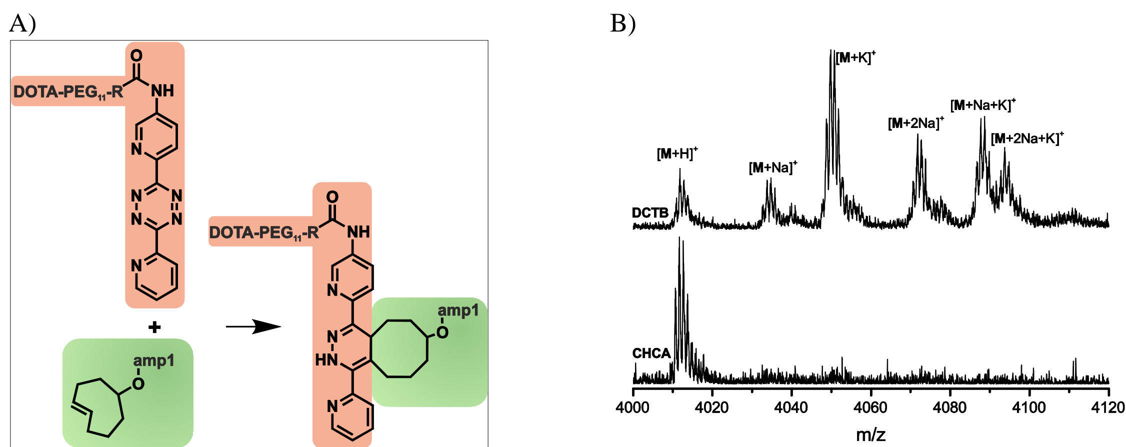
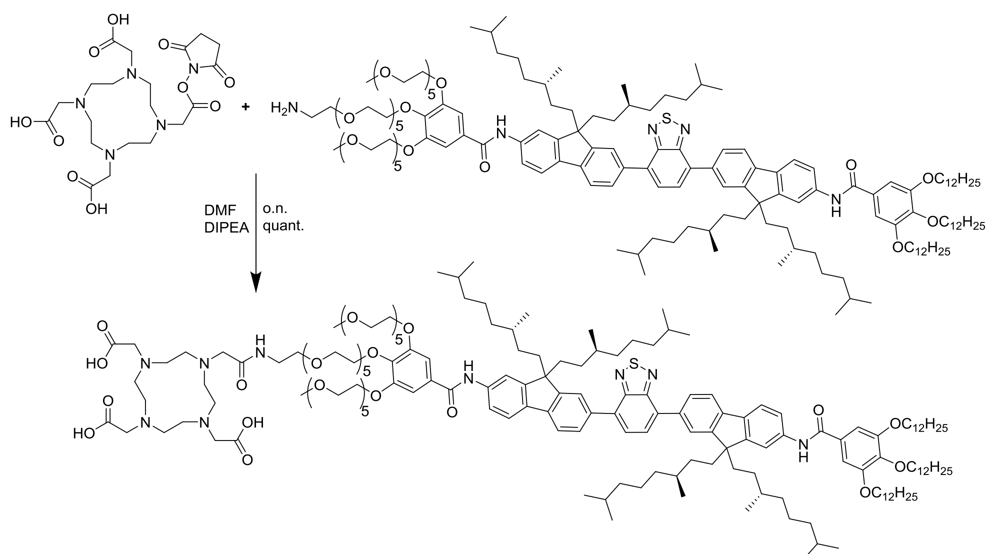


Fig. S2: A) Scheme of reaction between amp-TCO and tetrazine-DOTA, B) MALDI-ToF spectrum of the reaction mixture after 1 hour.

Table S2: Calculated and observed m/z values corresponding to MALDI-ToF spectrum of Fig. S2 B).

Species	**[M+H]$^+$**	**[M+Na]$^+$**	**[M+K]$^+$**	**[M+2Na]$^+$**	**[M+Na+K]$^+$**	**[M+2Na+K]$^+$**
Calc	4011.61	4033.58	4049.57	4055.59	4071.56	4093.55
Obs CHCA	4011.68	-	-	-	-	-
Obs DCTB	4011.82	4033.76	4049.86	4055.60	4071.81	4093.71

Amp-DOTA



Scheme S2: Synthesis of **amp**-DOTA

9.4 mg (4.6 µmol, 1 eq) of **amp**-NH_2[2] and 9.1 mg (15 µmol, 3.25 eq) NHS-DOTA were dissolved in 4 mL DMF containing 12 µL (20 eq) DIPEA. The mixture was stirred for 10 h, concentrated *in vacuo* and stirred up in THF. Supernatant was concentrated *in vacuo* and analysed by MALDI-ToF.

A) B)

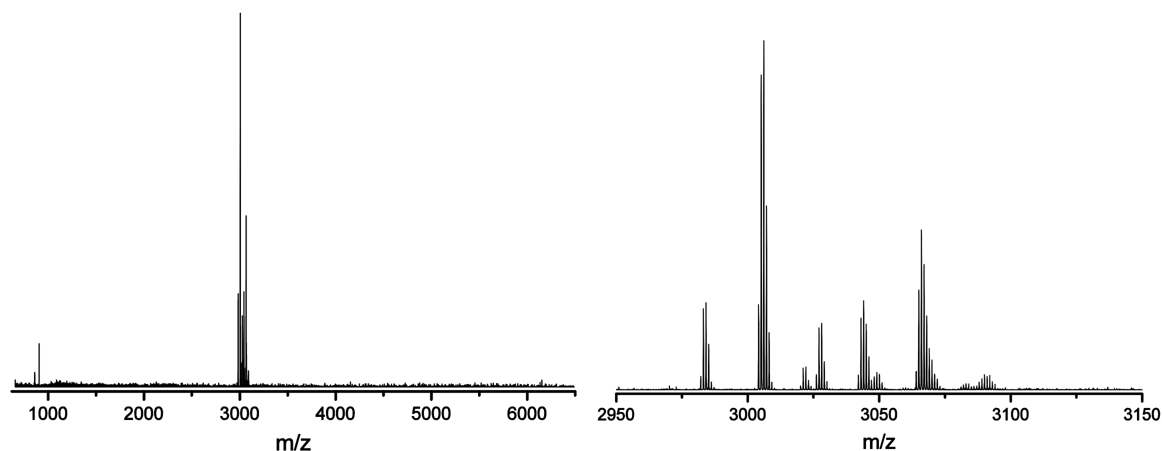
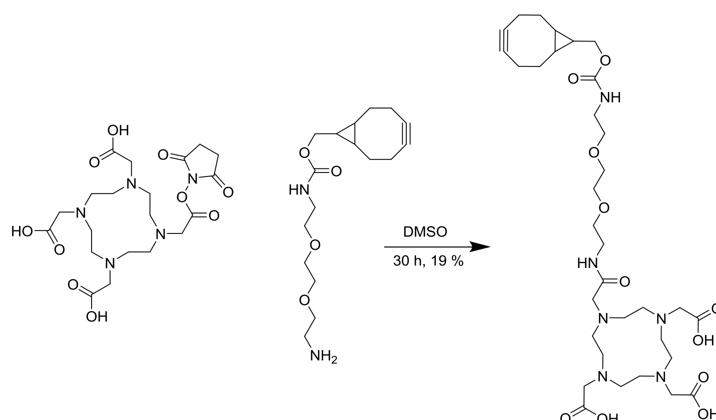


Fig. S3: A) and B) MALDI-ToF spectrum **amp**-DOTA.

Table S3: Calculated and observed m/z values corresponding to MALDI-ToF spectrum of Fig. S2 B).

Species	**[M+H]⁺**	**[M+Na]⁺**	**[M+K]⁺**	**[M+2Na]⁺**	**[M+Na+K]⁺**	**[M+2Na+K]⁺**
calc	2982.12	3004.09	3020.08	3026.10	3042.07	3064.06
obs	2982.12	3004.09	3020.07	3026.09	3042.04	3064.03

BCN-DOTA



Scheme S3: Synthesis of BCN-DOTA.

4.86 mg (15 µmol, 1 eq, 78.5 µL) of bicyclo[6.1.0]non-4-yn-9-ylmethyl (2-(2-(2-aminoethoxy)ethoxy)ethyl)carbamate (bought from Synaffix) was combined with 9.03 mg (18µmol, 1.2 eq) NHS-DOTA in DMSO (1 mL) and stirred for 30 h. Crude reaction mixture was concentrated *in vacuo* and purified by HPLC, obtaining product in 19 % yield (2.02 mg, 2.85 µmol). LC-MS analysis of the purified material proved that NHS-DOTA R_t = 1.08 min and BCN-NH_2 R_t = 4.16 min were not present and product Rt = 4.60 min appeared as a single peak.

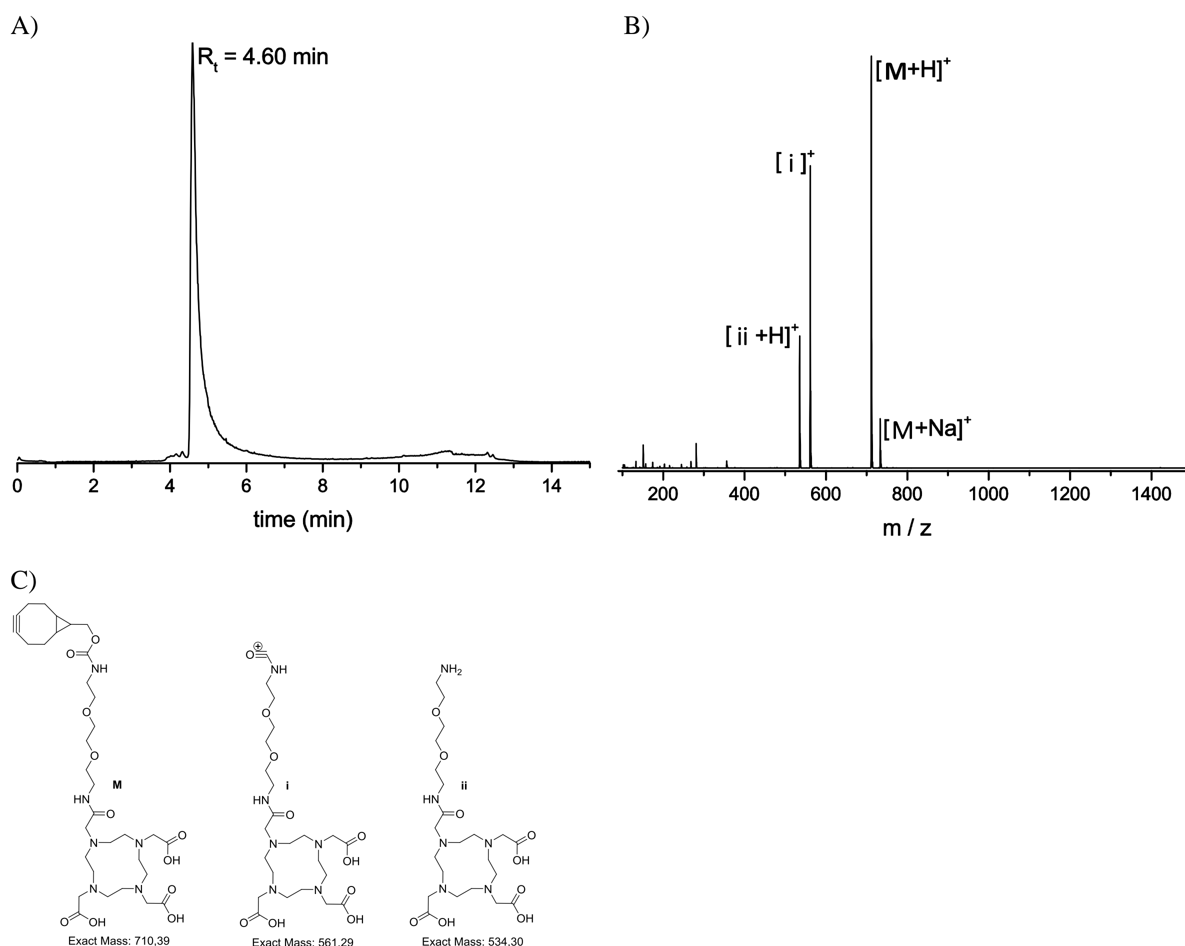


Fig. S4: LC-MS analysis of BCN-DOTA: A) TIC trace on 5 → 100% ACN / water gradient, B) MS spectrum at 4.60 min retention time, C) Chemical structures of observed MS species.

Table S4: Calculated and observed m/z values corresponding to MS spectrum of Fig. S3 B).

Species	**$[i+H]^+$**	**$[ii]^+$**	**$[M+H]^+$**	**$[M+Na]^+$**
Calc	535.30	561.29	711.39	733.38
obs	535.58	561.50	711.58	733.50

5. SMNP labeling strategy with amp-DOTA

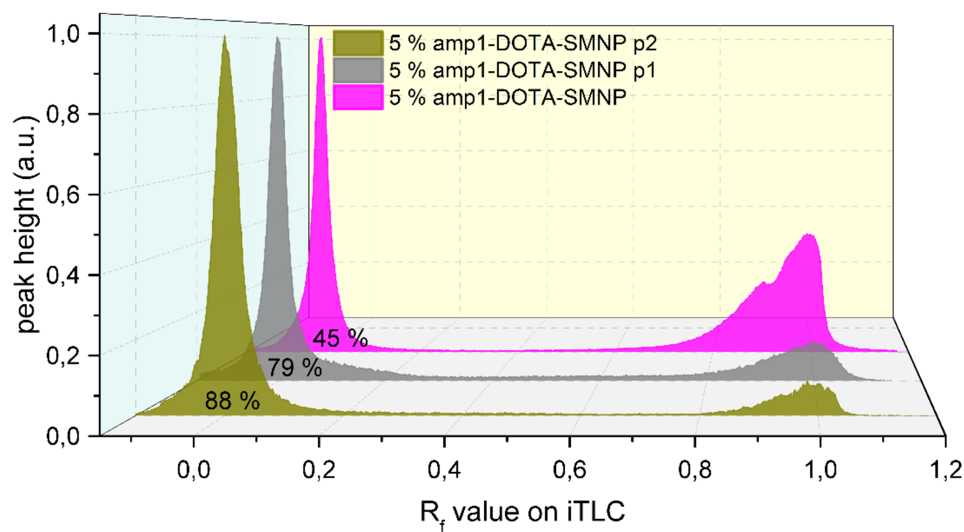


Fig. S5: iTLC of SMNPs containing 5 % **amp1**-DOTA, labeled in HEPES buffer for 35 min at 60°C, followed addition of excess DTPA competition for 5 min. The 5 % **amp1**-DOTA-SMNP system was purified by size exclusion column and result after one zeba (p1) or two zeba columns (p2) is presented.

6. SMNP labeling strategy with BCN-DOTA

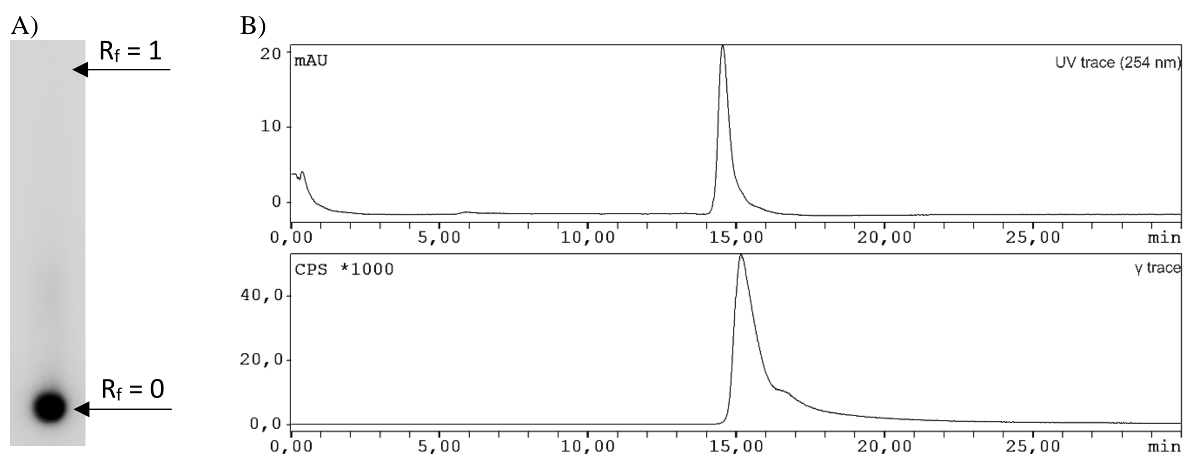


Fig. S6: BCN-DOTA was radiolabeled in 0.2 mM NH_4OAc buffer at pH 5.5 for 30 min at 60°C analysed by A) iTLC ($R_f = 0$ on bottom) and B) water-based size exclusion chromatography (SEC), top graph corresponds to the UV-trace (254 nm), bottom to the γ-trace with on the x-axis the retention time in min. Both techniques confirm complete labeling.

The reactivity of BCN-DOTA towards tetrazine was subsequently assessed by incubation of ^{111}In-labeled BCN-DOTA with an excess of tetrazine-coated-albumin[3] and analysed by water-based size exclusion chromatography. The tetrazine coated albumin absorbs UV-light at 254 nm and has a retention time on the water-based SEC of 12.8 min. If albumin-tetrazine reacts with ^{111}In-labeled BCN-DOTA, the γ-trace should reveal activity at the same retention time of albumin-tetrazine (12.8 min). In Figure S7A, the SEC trace of ^{111}In-labeled BCN-DOTA combined with albumin-tetrazine after 1 hour incubation is presented. The UV-trace showed the absorbance of albumin-tetrazine at 12.8 min, as well as the absorbance of ^{111}In-labeled BCN-DOTA at 14.7 min. The γ-trace revealed a peak at 15.2 min, most likely corresponding to BCN-DOTA, similar as in the figure above. The SEC-trace of the reaction mixture after 6 hours (Fig. S7B) was similar as the trace of 1 hour. No activity was observed at R_t = 12.8 min, which implies that no co-localization of tetrazine-albumin and BCN-DOTA occurred. This observation proved that no reaction between ^{111}In-labeled BCN-DOTA and albumin-tetrazine took place.

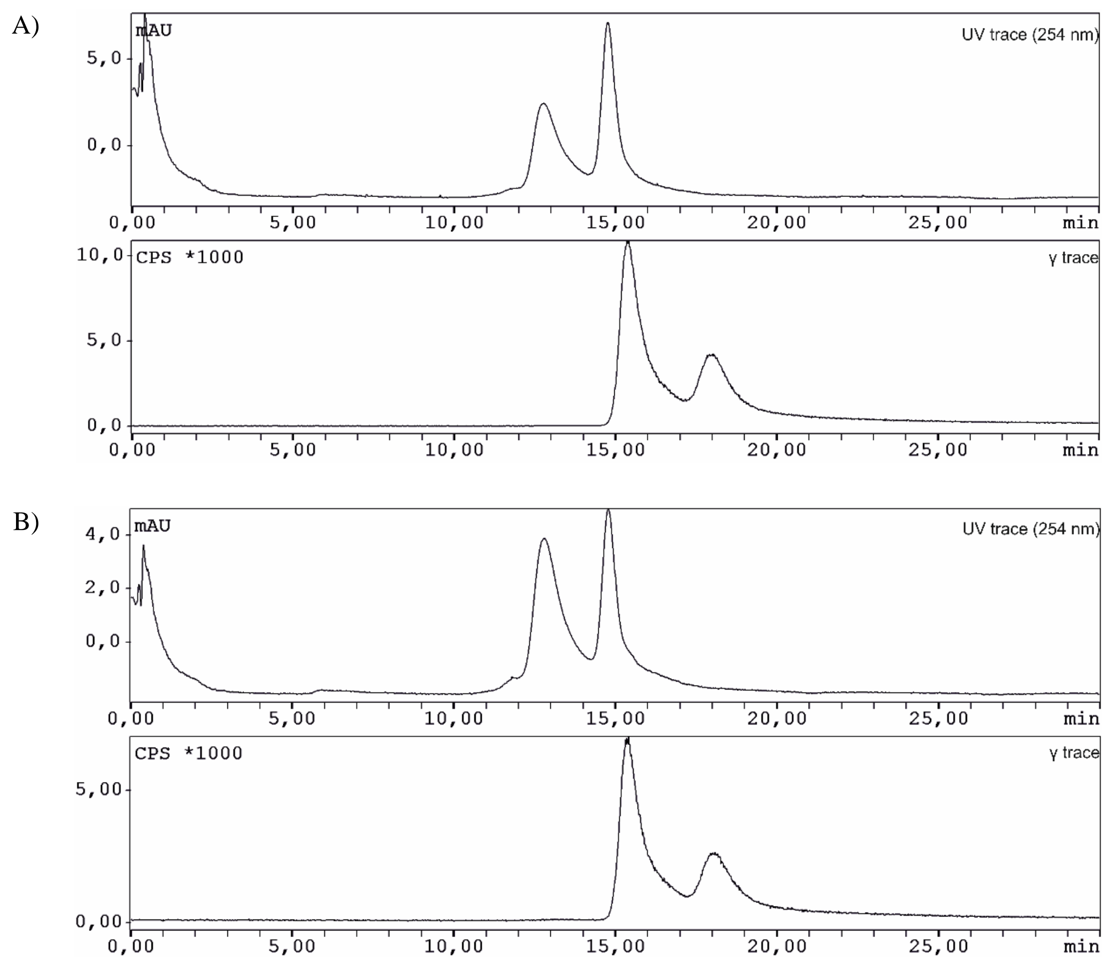
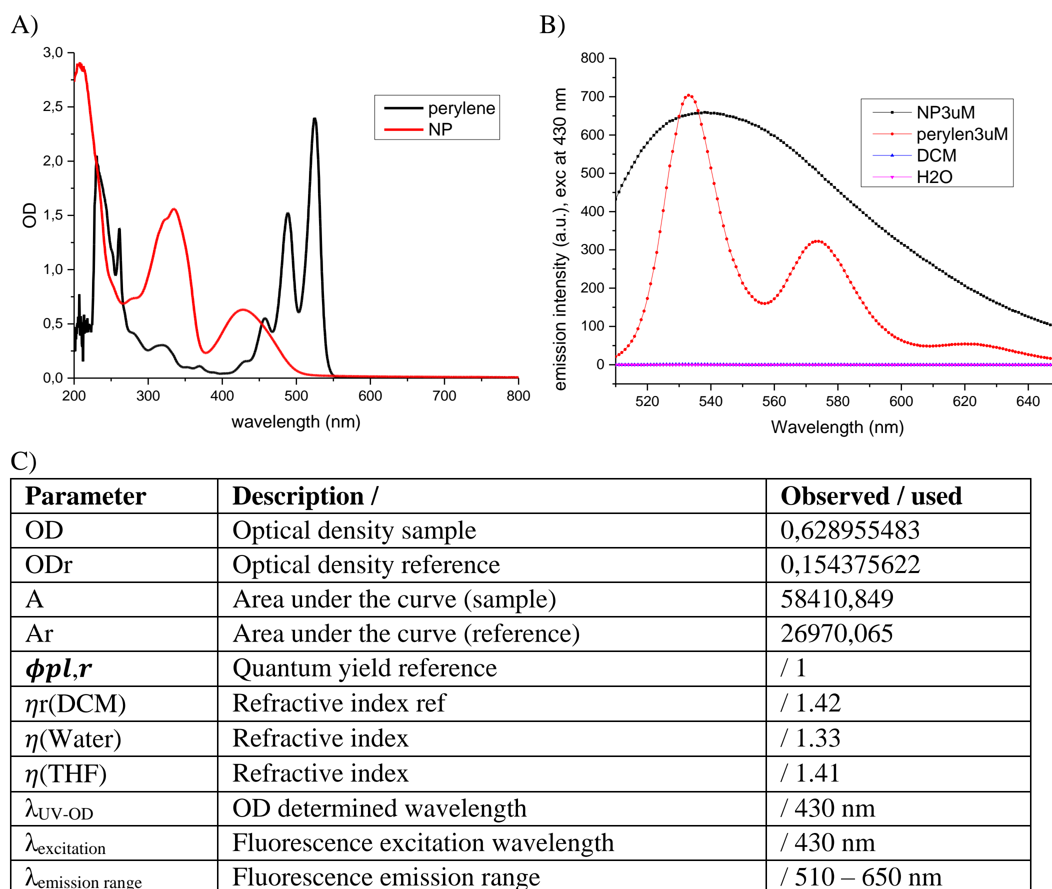


Fig. S7: A) SEC UV- and γ trace of ^{111}In-labeled BCN-DOTA combined with albumin-tetrazine 1 hour after incubation, B) SEC UV- and γ trace of ^{111}In-labeled BCN-DOTA combined with albumin-tetrazine 6 hours after incubation.

7. Physical properties of SMNPs and control studies

Quantum yield and absorption cross-section of SMNPs

The quantum yield was determined by the formula and data presented in figure S8. As a reference compound, N,N'bis(penthylhexyl) perylene bisimide was used, dissolved in DCM at 30 uM.



Parameter	Description /	Observed / used
OD	Optical density sample	0,628955483
ODr	Optical density reference	0,154375622
A	Area under the curve (sample)	58410,849
Ar	Area under the curve (reference)	26970,065
$\boldsymbol{\phi pl,r}$	Quantum yield reference	/ 1
ηr(DCM)	Refractive index ref	/ 1.42
η(Water)	Refractive index	/ 1.33
η(THF)	Refractive index	/ 1.41
$\lambda_{UV\text{-}OD}$	OD determined wavelength	/ 430 nm
$\lambda_{excitation}$	Fluorescence excitation wavelength	/ 430 nm
$\lambda_{emission\ range}$	Fluorescence emission range	/ 510 – 650 nm

D)

$$\varphi_{pl} = \varphi_{pl,r} \frac{A}{A_r} \frac{OD_r}{OD} \frac{\eta^2}{\eta_r^2} = 0.47$$

The absorption cross section (σ) was determined assuming a particle density of 1 g / cm^3,[2] and a particle size of 50 nm.

Fig. S8: A) UV-Vis absorption spectrum of perylene in dichloromethane (DCM) and 25% TCO-SMNPs in water, both at 30 µM, B) Fluorescence emission spectra of perylene in DCM and SMNPs in water at 3 µM, C) definitions of used parameters and used values for determining quantum yield, D) formula used for determining the quantum yield.

Control experiments of SMNPs

Several control studies with SMNPs were performed to investigate the influence of tz-DOTA ^{111}In-labeling on the fluorescence of 25% TCO-SMNPs, zeba size exclusion column purification

on fluorescence and size, SMNP serum incubation on fluorescence and the stability of 111-indium after reaction via tz-DOTA with TCO-SMNPs.

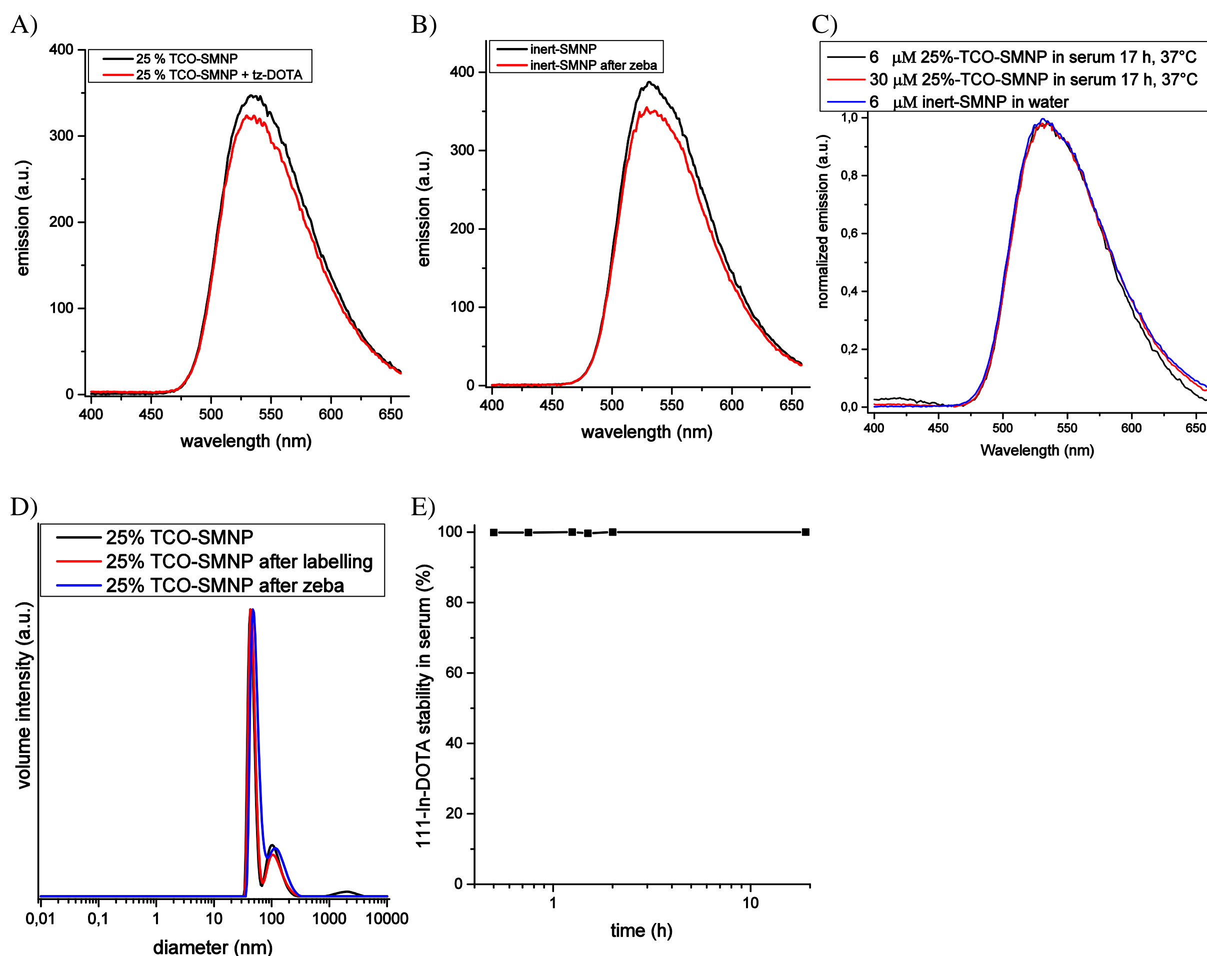


Fig. S9: A) fluorescence spectrum of 25 % TCO-SMNP before and after Tz-DOTA reaction, B) fluorescence spectrum before and after zeba (size exclusion column) purification, C) fluorescence spectrum of 25 % TCO-SMNPs incubed for 17 h at 37°C at 6 and 30 µM and **amp**-inert SMNPs at 6 µM, D) dyamic light scattering (DLS) data of 25 % TCO-SMNPs before ^{111}In-labeling, after ^{111}In-labeling with Tz-DOTA and after zeba (size exclusion column) purification, E) Stability of Indium-111 of labaled TCO-SMNPs by tz-DOTA.

SMNP detection from blood samples

Blood samples taken from the mice just after SMNP administration were collected in epps containing a small known amount of heparin to prevent coagulation. The blood samples were, after γ-counting, diluted 10 times with PBS and the fluorescent spectrum was recorded. By subtracting the fluorescent spectrum of blood samples taken two minutes after SMNP injection, with blood samples lacking SMNPs, the fluorescence component of SMNPs in blood could be obtained (Figure 3b).

8. References

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(2) Fischer, I., Petkau-Milroy, K., Dorland, Y. L., Schenning, A. P. H. J., and Brunsveld, L. (2013) Self-Assembled Fluorescent Organic Nanoparticles for Live-Cell Imaging. *Chem. – Eur. J. 19*, 16646–16650.
(3) Rossin, R., Läppchen, T., Bosch, S. M. van den, Laforest, R., and Robillard, M. S. (2013) Diels–Alder Reaction for Tumor Pretargeting: In Vivo Chemistry Can Boost Tumor Radiation Dose Compared with Directly Labeled Antibody. *J. Nucl. Med. 54*, 1989–1995.