Electronic Supporting Information

Functionalization of Theranostic AGuIX® Nanoparticles for PET/MRI/Optical Imaging

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Contents

1. Materials

All the chemicals used for the synthesis of multimodal platforms were purchased from ACROS, unless otherwise stated. Following is the list of reactants and their respective vendors: HOBt: Sigma-Aldrich; HBTU: Iris Biotech; Tris; DIPEA, $NaHCO_3$: Alfa Aesar; IR-783: Sigma-Aldrich; CH_2Cl_2: VWR. 4-(4,7-bis(2-(tert-butoxy)-2-oxoethyl)-1,4,7-triazacyclononan-1-yl)-5-(tert-butoxy)-5-oxopentanoic acid (NODAGA(tBu)$_3$) was obtained from CheMatech® and used without further purification. ^{64}Cu was obtained as Copper (^{64}Cu) chloride in 0.1N Hydrochloric acid (Cuprymina 925 MBq/mL) from SPARKLE S.r.l, Italy.

The SiGdNP nanoparticles were synthesized by a top-down process described by Mignot *et al.*[1]. Copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$, 98%) was purchased from Merck (France). Hydrochloric acid (HCl, 37%) was purchased from VWR Chemicals BDH Prolabo (France). Sodium hydroxide pellets (NaOH, ≥ 98%) were purchased from Sigma-Aldrich Chemicals (France). Solutions of hydrochloric acid and sodium hydroxide in water at different concentrations from 2 M to 10^{-4} M were prepared to adjust pH of solutions. Aminopropyltriethoxysilane ($H_2N(CH_2)_3$-$Si(OC_2H_5)_3$, APTES, 99%), Trifluoroacetic acid (TFA) analytical standard, glacial acetic acid for preparing buffer at pH 5 were purchased from Sigma-Aldrich Chemicals (France). Gadolinium chloride hexahydrate ($GdCl_3.6H_2O$, 99.999%) and europium chloride hexahydrate ($EuCl_3 \cdot 6H_2O$, 99.999%) were purchased from Metal Rare Earth Limited (China). CH_3CN for HPLC Plus Grade was purchased from Carlo Erba Reagents (France). Milli-Q water (ρ > 18 MΩ) was used as water source. Vivaspin™ concentrators (MWCO = 3 kDa or 5 kDa) were purchased from Sartorius Stedim Biotech (France). Gd (1000 mg/mL ± 0.2%) ICP single element standard solutions were purchased from Carl Roth (France). Radiochromatograms were carried out with a Raytest miniGITA-Star radiochromatograph (Raytest, Straubenhardt, Germany) or with a Bioscan AR-2000 radio-TLC Imaging Scanner (Bioscan Inc., Washington, DC).

2. Methods

2.1. Synthesis of trifunctional probe and functionalization of AGuIX

2.1.1. Synthesis of 6-Maleimidohexanoic-Lysine-Boc (1)

6-Maleimidohexanoic acid (204 mg, 0.95 mmol) was dissolved in CH_3CN (4 mL). DIPEA (0.18 mL, 1.0 mmol), then TSTU (305 mg, 1.0 mmol), were added to the solution. The solution was stirred at r.t. for 30 min and the NHS ester formation was checked by LC-MS. A solution of Boc-Lys-OH (236 mg, 0.95 mmol) in 4 mL of MeOH and DIPEA (0.34 mL, 1.9mmol) was pipetted into the maleimide solution. The reaction was stirred at r.t. for 1 h and the product formation was checked by LC-MS. The solvents were evaporated and the residue was purified by flash-chromatography (C18) using water (0.1% TFA) and CH_3CN (0.1% TFA) as eluents to yield compound (**1**) as a cream colored powder after lyophilisation (451 mg, 96%).^{1}H NMR (500 MHz, $CDCl_3$, 300K) δ(ppm): 1.2 – 1.3 (m, 2H), 1.4 (m, 10H), 1.5 – 1.7 (m, 4H), 1.8 (m, 1H), 2.1 (m, 2H), 3.1 – 3.2 (m, 2H), 3.5 (t, J = 7.2 Hz, 2H), 6.6 – 6.9 (m, 1H). ^{13}C NMR (126 MHz, $CDCl_3$, 300K) δ(ppm): 26.6, 29.1, 30.1, 32.0, 32.6, 35.8, 39.9, 41.5, 42.9, 57.1, 83.8, 138.0, 160.1, 175.1, 178.1, 178.7. HR-MS: m/z = Calculated for $C_{21}H_{33}N_3O_7Na$: 462.221 $[M+Na]^+$; Obtained: 462.222 $[M+Na]^+$. Elemental Analysis: Calculated for $C_{21}H_{33}N_3O_7$.

$2.5H_2O$(%): C: 52.06, H: 7.91, N: 8.67; Observed (%): C: 51.83, H: 7.49, N: 9.74. Analytical HPLC: t_r = 3.55 min, purity = 97%.

2.1.2. Synthesis of NODAGA(tBu)$_3$-NH$_2$ (2)

NODAGA(tBu)$_3$ (1 g, 1.84 mmol) was dissolved in CH_3CN (10 mL). DIPEA (1.3 mL, 7.36 mmol) was added first to the above solution followed by coupling agents viz. HBTU (0.75 g, 1.93 mmol) and HOBt (0.26 g, 1.93mmol), and the solution was stirred at r.t. for 10-15 min. A solution of ethylenediamine (6 mL, 89.8 mmol) in CH_3CN (10 mL) was added and the solution was stirred further at r.t. for 1h. The solvent was evaporated and the residue was dissolved in DCM (50 mL) and sequentially extracted with citric acid solution (50 mL, 0.1% w/v, pH 3), $NaHCO_3$ solution (50 mL, 5% w/v), and finally ultrapure water (50 mL) using a separating funnel to remove water soluble components. The organic phase was dried over $MgSO_4$, filtered and evaporated to yield a yellowish residue which was purified by reverse phase (C18) flash chromatography using CH_3CN (0.1% TFA): water (0.1% TFA) as eluents. The compound (**2**) was recovered as a white solid as a TFA salt (1134 mg, 68%). ^{1}H NMR (500 MHz, $CDCl_3$, 300K) δ(ppm): 1.5 (m, 27H), 1.9 – 2.2 (m, 2H), 2.4 (t, *J* = 7.3 Hz, 2H), 2.8 – 3.9 (m, 22H). ^{13}C NMR (126 MHz, $CDCl_3$, 300K) δ(ppm): 17.9, 21.5, 27.0, 27.6, 29.7, 30.2, 33.5, 38.3, 38.6, 38.9, 43.0, 45.8, 49.6, 50.7, 51.6, 53.0, 53.1, 54.0, 56.2, 61.8, 108.2, 169.9, 170.0, 174.9, 175.6, 175.7. HR-MS: m/z = Calculated for $C_{29}H_{56}N_5O_7$: 586.417 $[M+H]^+$; Obtained: 586.419 $[M+H]^+$. Elemental Analysis: Calculated for $C_{29}H_{55}N_5O_7$. 2.8TFA (%): C: 45.92, H: 6.44, N: 7.74; Observed (%): C: 45.31, H:6.30, N:7.55. Analytical HPLC: t_r = 3.84 min, purity = 98%.

2.1.3. Synthesis of 6-Maleimidohexanoic-Lysine-Boc-NODAGA(tBu)$_3$ (3)

6-Maleimidohexanoic-Lysine-Boc (**1**) (180 mg, 0.36 mmol) was dissolved in CH_3CN (5 mL). DIPEA (0.073 mL, 0.41 mmol), then TSTU (126 mg, 0.41 mmol) were added to the solution. The solution was stirred at r.t. for 1 h. A solution of NODAGA(tBu)$_3$-NH$_2$ (**2**) (240 mg, 0.27 mmol) in CH_3CN (10 mL) and DIPEA (0.29 mL, 1.64 mmol) was stirred at r.t. for 5 min and added to the activated lysine solution. The reaction mixture was stirred at r.t. for 2 h. The solvent was evaporated and the translucent residue was purified by reverse phase (C18) flash chromatography using water (0.1% TFA) and CH_3CN (0.1% TFA) as eluents. The compound (**3**) was recovered as a white solid after lyophilization as a TFA salt (280 mg, 55%). ^{1}H NMR (500 MHz, $CDCl_3$, 300K) δ(ppm): 1.2 – 1.4 (m, 4H), 1.4 (s, 38H), 1.6 (m, 5H), 1.7 – 1.8 (m, 2H), 2.0 – 2.1 (m, 1H), 2.1 (m, 1H), 2.2 (m, 2H), 2.4 (t, *J* = 7.2 Hz, 2H), 2.5 – 3.6 (m, 21H), 3.6 – 3.9 (m, 5H), 4.0 – 4.2 (m, 1H), 4.3 – 4.6 (m, 1H), 6.7 (s, 2H). ^{13}C NMR (126 MHz, $CDCl_3$, 300K) δ(ppm): 17.5, 22.6, 25.2, 26.2, 28.0, 28.0, 28.2, 28.3, 28.5, 32.2, 32.5, 36.1, 37.2, 37.6, 38.9, 39.1, 39.5, 50.4, 53.9, 54.7, 55.9, 63.5, 79.8, 83.1, 114.8, 117.1, 134.1, 160.6, 160.9, 170.7, 170.9, 173.1, 173.6, 174.2. HR-MS: m/z = Calculated for $C_{50}H_{87}N_8O_{13}$: 1007.638 $[M+H]^+$; Obtained: 1007.639 $[M+H]^+$. Elemental Analysis: Calculated for $C_{50}H_{87}N_8O_{13}$. 2TFA. $4H_2O$ (%): C: 49.61, H: 7.40, N: 8.57; Observed (%): C: 49.61, H: 7.56, N: 9.38. Analytical HPLC: t_r = 4.68 min, purity = 98.%.

2.1.4. Synthesis of 6-Maleimidohexanoic-Lysine-NODAGA (4)

6-Maleimidohexanoic-Lysine-Boc-NODAGA($tBu)_3$ (**3**) (180 mg, 0.14 mmol) was dissolved in TFA (3 mL). The solution was stirred at r.t. for 3 h, after which the reaction was deemed complete as verified by LC-MS. The TFA was evaporated and the resulting viscous residue was dissolved in DCM (5 mL) and re-evaporated to remove residual TFA. The residue was purified by flash chromatography (C18) using water (0.1% TFA) and CH_3CN (0.1% TFA) as eluents. The compound (**4**) was obtained as a cream white powder after lyophilisation of the appropriately identified and collected fractions (110 mg, 76%). ^{1}H NMR (500 MHz, D_2O, 300K) δ(ppm): 1.3 (m, 3H), 1.4 – 1.6 (m, 7H), 1.7 – 1.8 (m, 2H), 1.9 (m, 1H), 2.0 – 2.1 (m, 1H), 2.1 (t, *J* = 7.4 Hz, 2H), 2.3 – 2.4 (m, 2H), 3.0 (m, 9H), 3.1 – 3.3 (m, 15H), 3.3 – 3.5 (m, 4H), 3.5 (dd, *J* = 7.8, 6.5 Hz, 1H), 3.8 (s, 4H), 3.9 (t, *J* = 6.7 Hz, 1H), 6.8 (s, 2H). ^{13}C NMR (126 MHz, D_2O, 300K) δ(ppm): 21.5, 24.3, 24.9, 25.4, 27.3, 28.0, 30.4, 32.6, 35.6, 37.4, 38.5, 38.7, 38.7, 42.2, 50.8, 53.2, 55.8, 64.1, 115.1, 117.5, 134.3, 162.8, 163.0, 169.9, 173.3, 175.4, 175.4, 176.7. HR-MS: m/z = Calculated for $C_{33}H_{55}N_8O_{11}$: 739.398 $[M+H]^+$; Obtained: 739.400 $[M+H]^+$. Elemental Analysis: Calculated for $C_{33}H_{55}N_8O_{11}$. 2TFA. $2H_2O$ (%): C: 44.31, H: 6.03, N: 11.17; Observed (%): C: 43.85, H: 6.47, N: 11.29. Analytical HPLC: t_r = 2.6 min, purity = 90%.

2.1.5. Synthesis of IR-783-Hexanoic Acid (5)

IR-783 (56 mg, 0.07 mmol) was dissolved in MeOH (3 mL). Triethylamine (0.036 mL, 0.267 mmol) and 6-mercaptohexanoic acid (22 mg, 0.13 mmol) were pipetted into the dye solution and the reaction was stirred at r.t. overnight (16 h) protected from light. The solvents were evaporated and the resulting dark green residue was purified by semi-prep HPLC (C18) using water (0.1% TFA) and CH_3CN (0.1% TFA) as eluents. The fractions were identified, evaporated and lyophilized to yield compound (**5**) as a green powder (45 mg, 68%). HR-MS: m/z = Calculated for $C_{44}H_{57}N_2O_8S_3$: 837.328 $[M-2H]^-$; Obtained: 837.331 [M-2H]. Elemental Analysis: Calculated for $C_{44}H_{59}N_2O_8S_3$. $3H_2O$ (%): C: 59.10, H: 7.33, N: 3.13, S: 10.0; Observed (%): C: 58.89, H: 7.07, N: 2.72, S: 10.53. Analytical HPLC: t_r = 4.29 min, purity = 98%.

2.1.6. Synthesis of IR-783-Lys(Mal)NODAGA (6)

IR-783-Hexanoic acid (**5**) (24 mg, 0.03 mmol) was weighed into an eppendorf tube and dissolved in anhydrous DMF (0.5 mL). Triethylamine (0.015 mL, 0.108 mmol) and TSTU (8.5 mg, 0.03 mmol) were added to the solution. The reaction mixture was stirred at r.t. for 15 min and the formation of the NHS ester was checked by LC-MS. 6-Maleimidohexanoic-Lysine-NODAGA (**4**) (20 mg, 0.02 mmol) was weighed into an eppendorf tube and dissolved in a 0.5M borate buffer (0.5 mL). The pH was measured and adjusted to 7-8, if needed with a 2M NaOH solution. The aqueous solution was added into the DMF solution and the reaction mixture was stirred at r.t. for 15 min. The solution was purified by semi-prep HPLC to yield compound (**6**) as a green powder after lyophilization (15 mg, 32%). HR-MS: m/z = Calculated for $C_{77}H_{111}N_{10}O_{18}S_3$: 1559.723 $[M]^+$; Obtained: 1559.727 $[M]^+$. Analytical HPLC: t_r = 4.17 min, purity = 99%.

2.1.7. Synthesis of AGuIX-SH nanoparticles (thiolation of nanoparticles)

AGuIX (112 mg, 100 µmol in Gd) was redispersed at 200 mM in PBS buffer (pH of the buffer was adjusted to 8 by dil. NaOH solution). 2-iminothiolane (13.7 mg, 100 µmol) was dissolved at 300 $g \cdot L^{-1}$ in PBS buffer (pH 8). The 2-iminothiolane solution was added gradually under vigorous stirring to AGuIX solution. Then, 100 µL of PBS (pH 8) buffer was added. The final pH of the mixture was adjusted to 8 by adding dil. NaOH solution. Finally, PBS buffer (pH 8) was added to have a mixture of AGuIX and 2-iminothiolane both at 100 mM. The solution was stirred at r.t. for 1 h. The pH was adjusted to 7.3 by adding dil. HCl solution. Thiolated AGuIX (AGuIX-SH) was purified by tangential filtration (Vivaspin, MWCO-3kD) with a factor of 64 using PBS buffer (pH 6.8) as washing solvent to remove unreacted 2-iminothiolane and the corresponding hydrolyzed product. T_1, T_2 and D_H of the sample were measured and compared with original AGuIX. The concentration of purified sample in Gd was re-adjusted using the result of T_1 while assuming that the longitudinal relaxivity did not change during the thiolation reaction. The sample was quickly titrated with Ellman's reagent to quantify the amount of grafted thiols.

2.1.8. Synthesis of AGuIX-NODAGA-IR783 nanoparticles

AGuIX-SH purified solution (266 µL, approx. 45 µmol in Gd) was taken in a glass bottle. IR-783-Lys(Mal)NODAGA (**6**) (9 mg, 4.5 µmol) was dissolved in 50 µL PBS buffer (pH 6.8). Dil. NaOH solution was added to adjust pH to 6.5 to fully dissolve the fluorophore. The solution of fluorophore was transferred to AGuIX-SH solution gradually under vigorous stirring. Finally, 50 µL of PBS buffer (pH 6.8) were added to have the mixture with AGuIX-SH at approximately 100 mM and IR-783-Lys(Mal)NODAGA at 10 mM. The mixture was stirred at r.t. for 2 h. The final concentrations of AGuIX-SH and IR-783-Lys(Mal)NODAGA (**6**) were approximately 58.5 mM and 5.8 mM respectively. pH was not changed after the reaction. The functionalized nanoparticles were purified by tangential filtration with a factor of 256 using Vivaspin (MWCO-3kD) and then lyophilized to yield a green powder with a yield of around 50%. The absorption of filtrates was measured to verify the efficiency of purification process. T_1, T_2 and D_H of the sample were measured and compared with particles in previous steps. A sample of purified particle was also taken for UV-vis spectroscopy and HPLC analysis.

2.2. Dynamic light scattering (DLS) for hydrodynamic size determination

Hydrodynamic diameter distribution of the nanoparticles was measured by DLS with a Zetasizer Nano-S (633 nm He-Ne laser) from Malvern Instruments. Measurement was taken on 1 mL of the solution at around 5 – 10 $g \cdot L^{-1}$ with a single use PMMA cuvette (Carl Roth GmbH, Germany) at 20 °C. Attenuator and position were optimized by the device. Fast mode was enabled to enhance the precision for the measurement of particles with hydrodynamic diameter less than 10 nm.

2.3. High-Performance Liquid Chromatography (HPLC)

General system I: For chemical analysis

The purity of the compound was assessed by RP-HPLC on a Thermo Scientific Dionex Ultimate 3000 system with a photodiode detector according to the following method: RP-HPLC (Phenomenex Kinetex C_{18} column, 2.6 μm, 2.1 × 50 mm) with CH_3CN and H_2O + 0.1% TFA as eluents. 5 % CH_3CN (2 min), followed by linear gradient from 5 to 100 % (5 min) of CH_3CN, 100 % CH_3CN (1.5 min), a return to initial conditions by linear gradient from 100 to 5 % (0.1 min) and 5 % CH_3CN (1.4 min) at a flow rate of 0.5 mL·min^{-1}. UV-vis detection was achieved at four distinct wavelengths (201, 214, 220 and 254 or 700nm) in the range 200-700 nm (along with diode array detection within the same wavelength range).

General system II: For nanoparticles analysis

Gradient HPLC analysis was done by using the Shimadzu Prominence series UFLC system with a CBM-20A controller bus module, a LC-20AD pump, a CTO-20A column oven, and a SPD-20A UV−vis detector. The detecting wavelength was set at 295 nm for characterizing empty NP and at 700 nm where NODAGA copper complex specifically absorbs for characterizing copper incorporated NP. The column temperature was maintained at 30 °C. Gradient LC elution was carried out with two mobile phases: (A) ultrapure H_2O/TFA 99.9:0.1 v/v and (B) CH_3CN/TFA 99.9:0.1 v/v. Each time, an amount of 20 µL of sample was loaded to an injection valve and injected into a Jupiter C_4 column (150 mm × 4.60 mm, 5 µm, 300 Å, Phenomenex) at a flow rate of 1 mL·min^{-1}. Then the elution was programmed as follow: 1% of solvent B in 7 min to elute the reactive and fragments, then a gradient from 1% to 90% in 15 min to elute the nanoparticles. The concentration of B was maintained over 7 min. Then, the concentration of solvent B was decreased to 1% over 1 min and maintained during 8 min to re-equilibrate the system for a new analysis. Before the measurement of each sample, a baseline was obtained under the same conditions by injecting ultrapure water. The purity is calculated by dividing the area under the peak of the particle to the total area under the peaks of the particle and the reactive.

Chromatographic analysis of nanoparticles is quite unusual and daunting task. Nonetheless, HPLC analysis of the nanoparticles revealed valuable information upon the structural changes caused to the nanoparticles (Figure S1). AGuIX® nanoparticles typically exhibit a retention time of 13 min at 295 nm. Upon grafting the nanoparticle retention time shifted to right with elution at 18 min, and this could be due to increase in its hydrophobicity. The presence of the small peak before the main peak of functionalized nanoparticles could be due to residual underivatized nanoparticles. Furthermore, this was verified by performing the analysis at 700 nm which clearly demonstrated the functionalization of the nanoparticles.

2.4. Relaxivity measurements

Relaxivity measurements were performed on a Bruker® minispec mq60NMR analyzer (Brucker, USA) at 37 °C at 1.4 T (60 MHz). Samples were measured at a specific Gd^{3+} concentration (mM), measured from ICP-OES. The longitudinal relaxation time T_1 and the

transverse relaxation time T_2 (s) were measured. Then the relaxivities r_i ($s^{-1}.mM^{-1}$) (i = 1, 2) were obtained according to the following formula:

$$\left(\frac{1}{T_i}\right)measured = \left(\frac{1}{T_i}\right)water + r_i[Gd^{3+}]$$

$$\left(\frac{1}{T_i}\right)water \approx 0.2816\ (s^{-1})$$

$$i = 1\ or\ 2$$

2.5. Elemental analysis

The determination of the accurate concentration of gadolinium in the nanoparticles was performed by inductively coupled plasma - optical emission spectrometry (ICP-OES) (with a Varian 710-ES spectrometer). The solution after DLS measurement was reused for this measurement. The solution of particles at an estimated concentration in Gd of 10 ppm was digested for 3 h in 4-5 mL of aqua regia (HNO_3 67% mixed with HCl 37% (1 : 2, v/v)) at 80 °C. Subsequently, the mixture was diluted to estimated 100, 200 and 400 ppb at precisely 50 mL with HNO_3 5% (v/v). These solutions were filtered through 0.2 µm membrane before being analyzed. Calibrated samples were prepared from 1000 ppm Gd standard solution by successive dilutions with HNO_3 5% (w/w). The result was the average of the three samples at presumably 100, 200 and 400 ppb. Otherwise, elemental analysis was also conducted by FILAB SAS, Dijon, France and enabled determination of the Gd, C, N and Si contents of the powder samples.

2.6. Photophysical Characterization

UV-visible spectra were obtained on a Varian Cary 50 scan (single-beam) spectrophotometer by using a rectangular quartz cell (Hellma, 100-QS, 45 × 12.5 × 12.5 mm, pathlength 10 mm, chamber volume: 3.5 mL), at 25 °C (using a temperature control system combined with water circulation). Fluorescence spectra (emission/excitation spectra) were recorded with an HORIBA Jobin Yvon Fluorolog spectrophotometer (software FluorEssence) at 25 °C (using a temperature control system combined with water circulation), using a standard fluorometer cell (Labbox, LB Q, 10 mm). Emission spectra were recorded in the range 755-900 nm after excitation at 740 nm (shutter: Auto Open, excitation slit = 5 nm and emission slit = 5 nm). Excitation spectra were recorded in the range 400-825 nm after emission at 840 nm (shutter: Auto Open, excitation slit = 5 nm and emission slit = 12 nm). All fluorescence spectra were corrected until 850 nm. Fluorescence quantum yields were measured at 25 °C by a relative method using ICG (Φ_F = 10.6% in DMSO) as a standard (dilution by a factor of 3 between absorption and fluorescence measurements) [2]. The following equation was used to determine the relative fluorescence quantum yield:

$$\Phi_F(x) = (A_S/A_X)(F_X/F_S)(n_X/n_S)^2\ \Phi_F(s)$$

where A is the absorbance (in the range of 0.01-0.1 A.U.), F is the area under the emission curve, n is the refractive index of the solvents (at 25 °C) used in measurements, and the subscripts s and x represent standard and unknown, respectively. The following refractive index values were used: 1.479 for DMSO and 1.337 for PBS. Stock solutions (1.0 $mg.mL^{-1}$) of **6** was prepared in ultrapure water and subsequently diluted with PBS for UV-vis absorption and fluorescence measurements. Stock solutions of **AGuIX-NODAGA-IR783** were prepared in PBS.

2.7. Radiochemistry and stability of radiolabelled nanoparticles

Radiolabelling: AGuIX-NODAGA-IR783 (2.8 mg) nanoparticles were dispersed into 13.3 µL of 1.0 M NH_4OAc buffer pH 5.8 and 13.3 µL of $^{64}CuCl_2$ (5.6 MBq) in 0.1 N HCl in an Eppendorf-type microtube. The nanoparticles dispersion was incubated at 37 °C for 30 min. The radiochemical purity was established by ITLC using 0.1 M EDTA as a mobile phase.

Plasma Stability: 26.6 µL of the radiolabelled dispersion of nanoparticles was diluted with 43.4 µL of PBS. 10 µL of the resulting solution was mixed with 100 µL of human plasma in an Eppendorf-type microtube. This tube was incubated at 37 °C for 48 h and aliquots were withdrawn at 24 h and 48 h to test the stability of the radiolabelled nanoparticles using ITLC.

EDTA Challenge stability: 26.6 µL of the radiolabelled dispersion of nanoparticles were diluted with 43.4 µL of PBS. 10 µL of the resulting solution was mixed with 100 µL of a buffer containing EDTA (50 mM) and HEPES (0.5 M) pH 7 in an Eppendorf-type microtube. This tube was incubated at 37 °C for 48 h and aliquots were withdrawn at 24 h and 48 h to test the stability of the radiolabelled nanoparticles using ITLC.

2.8. Animal Studies

Female NMRI Nude mice (6-8 weeks, Janvier Labs, France) were subcutaneously injected with $2x10^7$ TS/A cells (murine mammary adenocarcinoma). Mice were maintained in ventilated housing units under controlled conditions of temperature (22 ± 2°C), photoperiod (12h light/12h dark) with free access to food and drink. Three to five weeks after tumor cells injection, TSA tumor bearing-mice (n=3) were given 10mg of **AGuIX-NODAGA-IR783** (^{64}Cu) in PBS corresponding to radioactivity of 10.2 ± 0.3 MBq by intravenous injection. For biodistribution, at the end of 24h, animals were euthanized by isoflurane anesthesia followed by pentobarbital overdose. Blood, tumour and organs were collected, weighed and radioactivity in these samples was measured with a scintillation gamma – counter (Cobra 4180, Perkin Elmer, Waltham, MA, USA).

2.9. PET/MRI/Optical Imaging

Optical imaging was performed using the Perkin Elmer Ivis Lumina III system. Whole body imaging was acquired at 1h and 24h time points using 760/845 nm filters to get access to the « spectral un-mixing » tool (guided method) for autofluorescence background correction. All imaging data were analyzed with the Living Image® software. Values are reported as radiant efficiency allowing direct comparison of images from each experiment. *Ex vivo* imaging at 24h post injection was performed on excised tissues. Fluorescence intensities were

measured and normalized to photon per second per cm^2 using the region of interest (ROI) function of the Living Image® software. The ROI cover the entire tissue.

The PET-MR dual imaging was performed at 1 and 24 hours after the injection of the radiolabelled nanoparticles using a LabPET scanner (Trifoil Imaging) coupled in line with a small animal 3T MR small animal imaging system (MR Solutions) after anaesthesizing the mice with isofluorane (1.5-3% in air) [3]. After the last image acquisition, animals were sacrificed by isoflurane anesthesia followed by pentobarbital overdose. Blood, tumour and organs were collected, weighed and radioactivity in these samples was measured with a gamma- well counter. The counter was cross-calibrated to the dose calibrator used to measure the injected dose and the linearity range was determined for all the geometries used in ex-vivo counting. Data were then converted to percentage of injected dose and to percentage of injected dose per gram of tissue.

3. Figures and Tables

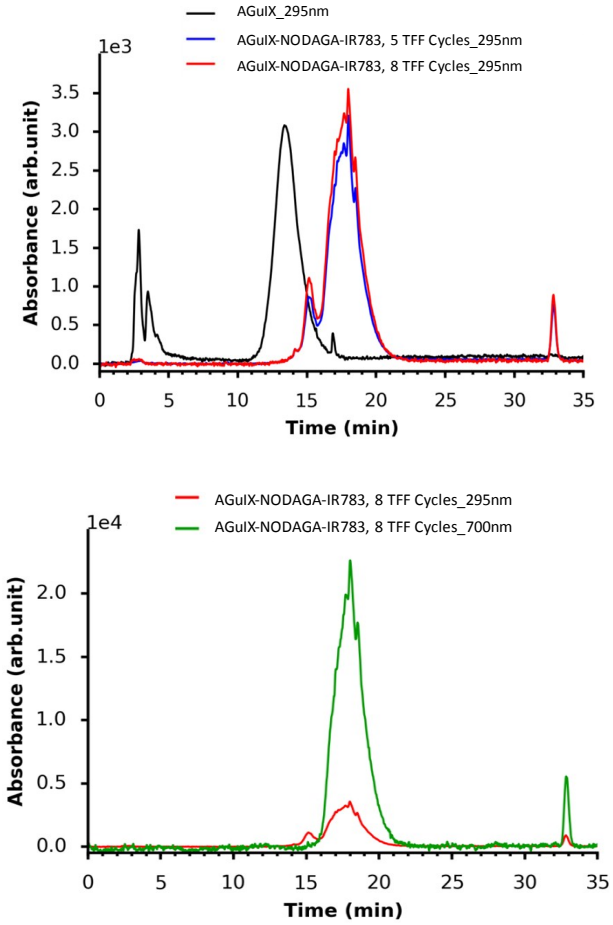

Figure S1. HPLC analysis of the AGuIX and functionalized nanoparticles at 295nm and 700nm.

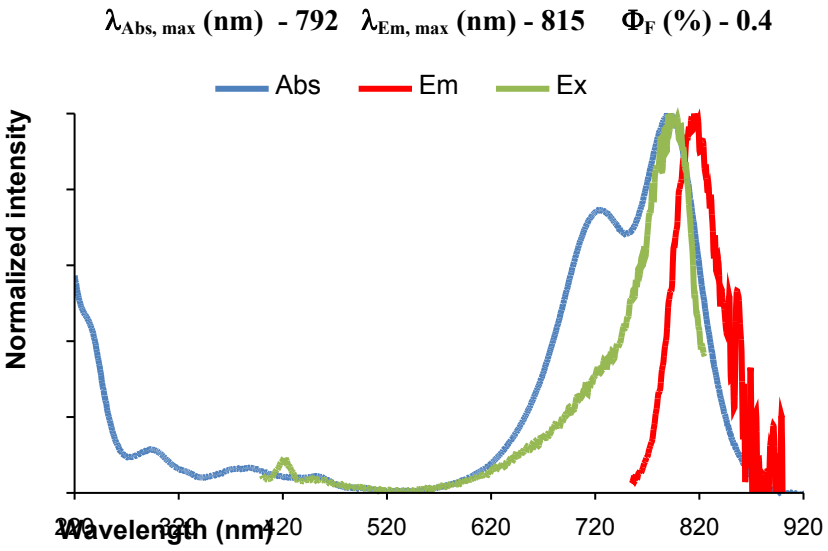

Figure S2. Photophysical properties of IR-783-Lys(Mal)NODAGA (6)

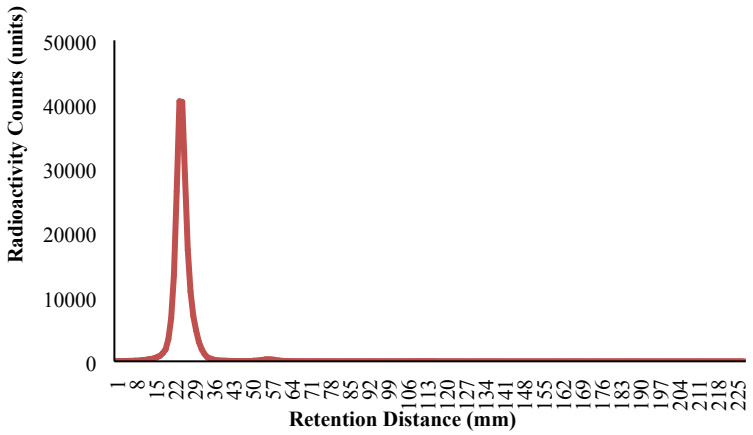

Figure S3. Radio chromatogram of the ^{64}Cu labelled AGuIX-NODAGA-IR783 obtained after ITLC-SA using 0.1M EDTA as a mobile phase

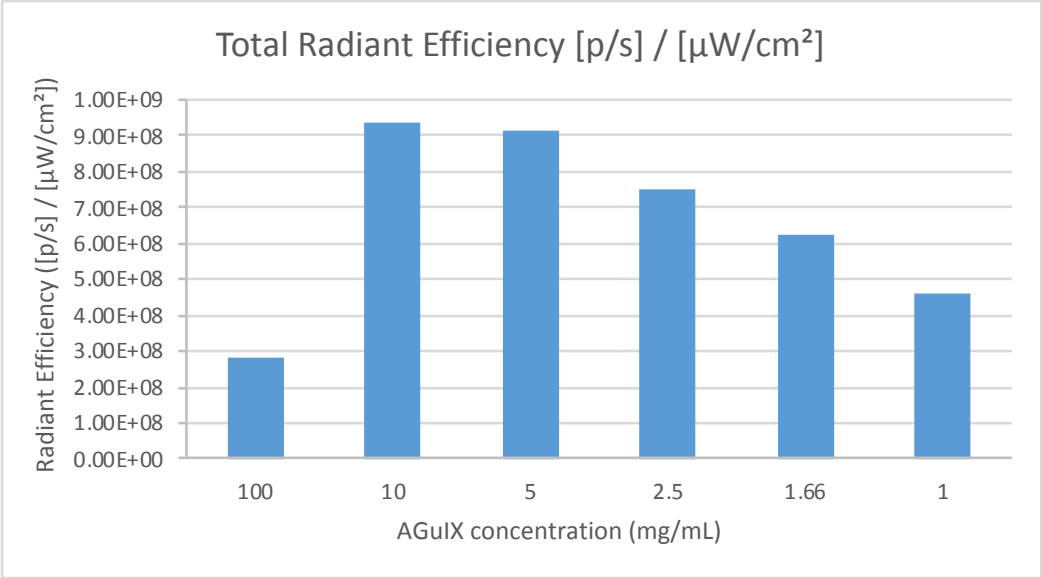

Figure S4. Total radiant efficiency of AGuIX-NODAGA-IR783 in saline solution at different concentrations: 100 mg/mL (injection concentration), 10, 5, 2.5, 1.66, and 1 mg/mL.

Table S1. Summary of the relaxometric characteristics of the nanoparticles

Sample	Concentration (mM)	T1	r_1	T2	r_2	r_2/r_1
AGuIX (FR30)	200	0.37	13.51	0.274	18.25	1.35
AGuIX-SH	70.78	0.84	16.82	0.604	23.39	1.39
AGuIX-NODAGA-IR-783	45.83	1.29	16.91	0.705	30.94	1.83

4. References:

[1] Mignot A, Truillet C, Lux F, Sancey L, Louis C, Denat F, Boschetti F, Bocher L, Gloter A, Stéphan O. et al., *Chem. Eur. J.* **2013**, 19, 6122-6136.

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[3] Vrigneaud J-M, Walker P, Barbier B, Camacho A, Oudot A, Collin B, Brunotte F, *Biomed. Phys. Eng. Express* **2017**, 3, 035006.