ADVANCED FUNCTIONAL MATERIALS

Supporting Information

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Cation Exchange Protocols to Radiolabel Aqueous Stabilized ZnS, ZnSe, and $CuFeS_2$ Nanocrystals with ^{64}Cu for Dual Radio- and Photo-Thermal Therapy

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S1 Materials and Method

Amino-functionalized polyethylene glycol methoxy terminated (NH_2-PEG2000-OCH_3, MW 2000 g/mol) was purchased from RAPP polymere. All the other reagents were purchased from Sigma-Aldrich and were used without further purifications.

The elemental analysis was performed using an Inductively-Coupled Plasma Atomic Emission Spectrometer (ICP-OES, ThermoFischer, CAP 6000). The samples were digested using a proper amount of aqua regia (HNO_3/HCl 1:3 ratio in volume) overnight, and they were diluted in order to obtain a 10% aqua regia solution. In case of biological samples (e.g. containing human serum) a digestion using a mixture of H_2O_2/HNO_3 1:2 was performed under sonication at 60 °C for 90 min. HCl was then added and sample diluted in order to obtain a 10% aqua regia solution.

TEM imagines were collected by a JEOL 1011 transmission electron microscope operating at an accelerating voltage of 100 kV. The TEM samples were prepared by drop-casting the solution onto a carbon coated copper grid and letting the solvent evaporate. For samples deposited from water, the TEM grid was left to dry overnight.

X-Ray Diffraction (XRD) patterns were recorded on a PANalytical Empyrean X-ray diffractometer equipped with a 1.8 kW Cu Kα ceramic X-ray tube and a PIXcel3D 2x2 area detector, operating at 45 kV and 40 mA. The diffraction patterns were recorded in air at room temperature using Parallel-Beam (PB) geometry and the symmetric reflection mode. The samples were prepared by drop casting the concentrated NC water solution onto a zero diffraction quartz substrate. This was followed by a drying step, which was conducted under a reduced pressure. The XRD data analysis was performed using the HighScore 4.6 software from PANalytical.

The hydrodynamic diameter of the NCs was determined by DLS measurements on a Mavern Zetasizer (Nano Series, Nano ZS) instrument. The scattered intensity was at 173° back scattered geometry with a 632 nm laser source. For each sample, three independent measurements were taken and each was the average of 10-15 acquisitions.

S2 Calculation of specific activity

The specific activity of the radiolabeled materials which are reported in **Table S1** was calculated taking into account the amount of material that was used in the radiolabeling experiments, the initial activity and radiochemical yield (RCY).

$$Specific\ activity\ (TBq/g) = \frac{Activity\ (TBq)}{mass\ of\ NCs\ (g)} \times RCY \qquad \text{Eq. S1}$$

For the materials that are reported in this work, we determined the RCY from the radio TLC integration reported in **Figure S9**. The amount of materials was calculated based on both the concentration of the NCs and the molecular weight of each NC (see below for more details on the NC concentration calculation).

S3 Nanocrystal synthesis

ZnSe NCs were synthesized accordingly to a previous published work.[1] In a typical synthesis 63 mg of zinc stearate, 54 mg of octadecylamine (ODA), 3.13 mL of squalane and 2.5 mL of octadecene (ODE) were added to a 25 mL three–necked flask. The mixture was heated to 120 °C and degassed under vacuum for 1 h. After switch to argon atmosphere, the temperature was set to 330 °C and reached in about 10 minutes. A selenium precursor was prepared in a 25 mL by dissolving 91 mg of selenium in 7 mL of oleylamine (OA) in a three-necked flask equipped with a condenser and under magnetic mixing. The mixture was degassed at 80 °C for 1h. Upon switching to argon atmosphere, the temperature was raised to 240 °C for 2 h until all of the selenium powder dissolved. Three mL of the Se solution was quickly injected into the Zn solution. The reaction was stopped after 20 min by removing the heating mantle. Dried toluene (3 mL) was injected, and the solution was transferred into a 20 mL scintillation vial equipped with septa. Dried isopropanol (10 mL) was added, and the NCs were collected upon centrifugation (3500 rpm, 5 min). The NCs were then re-dispersed in a minimum amount of toluene and precipitate (3 mL of dried ethanol) before centrifugation (3500 rpm, 5 min). This purification step was repeated twice. Finally, the NCs were dissolved in 3 mL of dried toluene. The Zn and Se content was determined *via* ICP-OES.

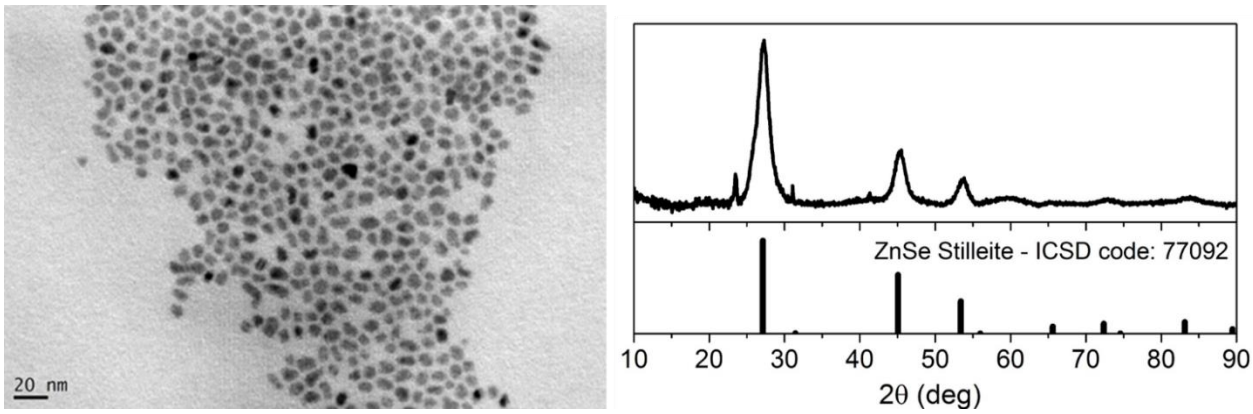


Figure S1 Representative TEM (scale bar is 20 nm) and XRD of as-synthesized octadecylamine coated ZnSe NCs .

$CuFeS_2$ NCs were synthetized following a protocol that has already been reported by our group.[2]

ZnS NCs were synthesized following the hot-injection protocol reported by Joo *et al.*[3] with minor modification. Briefly, 2.3 g of tri-octyl phosphine oxide (TOPO), 10 mL of OA and 273 mg (2 mmol) of $ZnCl_2$ were added to a 50 mL three necked flask connected to a standard Schlenk line set-up and equipped with a magnetic stirrer. The mixture was heated to 120 °C and degassed under vacuum for 90 min. After switch to nitrogen atmosphere, the temperature was raised to 290 °C. A sulfur precursor was prepared in a 25 mL three necked flask, equipped with a magnetic stirrer and connected to a Schlenk line, by dissolving 92 mg (6 mmol) of sulfur powder in 6 mL of OA. The solution was degassed at 80 °C for 90 min, then, it was cooled down to RT so that it could be injected into the $ZnCl_2$ solution. Soon after injection, the

temperature dropped to 240 °C. After few minutes, it reached 290°C again, and the color changed from opalescent yellow to clear red. The reaction was stopped after 60 minutes by removing the heating mantle. A minimum amount of hexane was added in order to dissolve the NCs, then an excess of ethanol was added as an anti-solvent (ratio hexane:ethanol 1:4). The NCs were collected by centrifugation (4500 rpm, 10 min). The washing step was repeated three times. The NCs were then dissolved in 20 mL of hexane, and 1 mL of OA was added in order to produce a colloidally stable suspension. The Zn and S content was determined *via* ICP-OES.

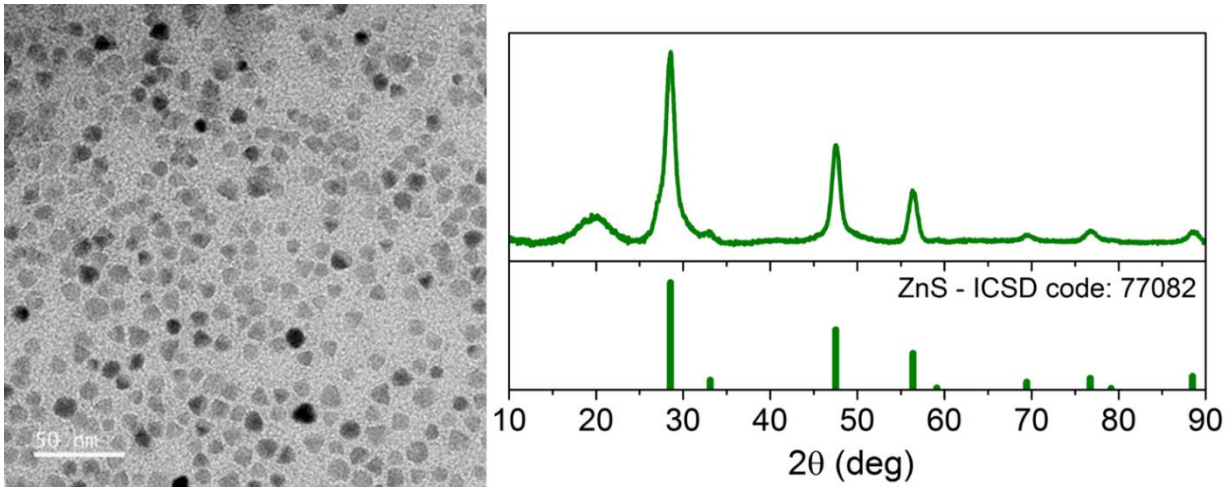


Figure S2. Representative TEM image (scale bar is 50 nm) and XRD of as-synthesized oleylamine coated ZnS NCs.

S4 Determination of nanocrystal concentration

The NC moles and concentration can be simply calculated by dividing the total mass of the elements (Zn and Se for ZnSe, Zn and S for ZnS and Cu, Fe, S for $CuFeS_2$) found in the solution by the Molecular Weight (MW) of a single NC. The MW of a NC is calculated from the mass of a single NC, which is obtained by multiplying the average volume of a NC (from the average dimension obtained at TEM analysis) by the bulk density obtained from the XRD analysis.

$$mass\ of\ single\ NC\ (g) = density\ (g/cm^3) \times volume\ single\ NC\ (cm^3) \quad \text{Eq. S2}$$

$$MW\ of\ single\ NC\ (g/mol)\ = mass\ of\ single\ NC\ (g) \times N_A(mol^{-1}) \quad \text{Eq. S3}$$

	ZnSe Spherical	**ZnS Spherical**	**$CuFeS_2$ tetrahedron**
Diameter / edge (nm)	6.0	10.0	12.6
Volume (nm^3)	113	523	235
Density (g/cm^3)	5.21	4.09	4.18
ICSD card	98-007-7092	98-007-7082	96-901-5235
Mass of single NC (g)	5.89×10^{-19}	2.14×10^{-18}	9.82×10^{-19}
MW of NC (g/mol)	354700	1288700	591400

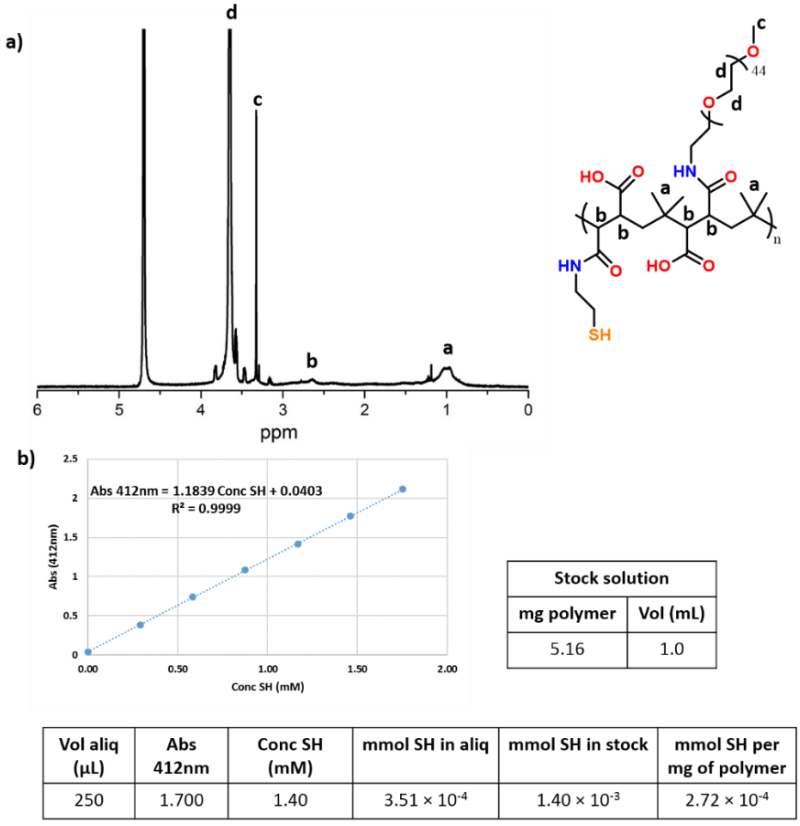
Table S1 Characteristics of ZnSe, ZnS and $CuFeS_2$ NCs.

The concentration of the nanocrystal solution can then be calculated from the mass of all the elements, the MW of the NC, and the volume of the solution as in **Eq S4**.

$$Conc\ of\ NCs\ (mol/L) = \frac{total\ mass\ of\ elements\ (g)}{MW\ of\ single\ NC\ (g/mol)} \Big/ Volume\ (L) \quad \text{Eq. S4}$$

S5 Cys-PIMA-PEG synthesis

Cys-PIMA-PEG were synthesized based on a procedure that has already been reported in literature,[4] with some modifications. 46 mg of poly(isobutylene-*alt*-maleic anhydride) PIMA (MW 6000 g/mol, 7.7 µmol, 0.3 mmol of monomer units) and 2.5 mL of DMSO were placed into a 8 mL glass vial equipped with a rubber septa. PIMA was dissolved by being gently heated up, then it was degassed by nitrogen bubbling for 10 min. The solution was heated up to 45 °C in an oil bath, then cysteamine hydrochloride (MW 113.61 g/mol, 17 mg, 0.15 mmol) dissolved in 1 mL of DMSO in presence of 21 µL of triethyl amine, was added dropwise. After 20 min, a NH_2-PEG-OCH_3 (MW 2000 g/mol, 0.15 mmol) solution, dissolved in 1 mL of DMSO, was added dropwise and the mixture was left to react overnight. The solution was left to cool down at RT, and it was stored at -20 °C. A characterization of the polymer was performed by means of proton NMR analysis and an Ellman's test (5,5'-Dithiobis(2-nitrobenzoic acid), following the supplier (Thermo-Fisher) indicated protocol for the quantification of sulfhydryl groups. For characterization purposes, the polymer that was dissolved in DMSO, then, diluted with DI water (1:4) and washed three times with water in an amicon centrifuge tube (Millipore Merck) with a MW cutoff of 3 kDa in order to remove all DMSO. The polymer was then lyophilized, yielding a white powder.



Stock solution	
mg polymer	Vol (mL)
5.16	1.0

Vol aliq (µL)	Abs 412nm	Conc SH (mM)	mmol SH in aliq	mmol SH in stock	mmol SH per mg of polymer
250	1.700	1.40	3.51×10^{-4}	1.40×10^{-3}	2.72×10^{-4}

Figure S3 a) ^{1}H-NMR spectra of Cys-PIMA-PEG in D_2O. b) Calibration curve from the Ellman test, and the sulfhydryl content of the cys-PIMA-PEG polymer.

S6 Water transfer: Cys-PIMA-PEG and SH-PEG-OCH_3 ligand exchange

ZnSe was transferred into water using cys-PIMA-PEG in the presence of $Zn(NO_3)_2$. In a 40 mL vial, 1 mL of ZnSe toluene solution (5.6 nm, 13µM) was precipitated using a slight excess of methanol. It was then centrifuged and collected as a dry pellet. The NCs were re-dispersed in 6 mL of chloroform. In a different vial, 650 µL of a cys-PIMA-PEG ligand in DMSO (77.8 mg polymer containing 0.021 mmol of –SH group, corresponding to about 17 SH/nm^2) was mixed with 60 mg of $Zn(NO_3)_2$•$6H_2O$. The ligand solution was then mixed with the NC solution. The ligand exchange solution was further heated to 55 °C for 30 min in a sonication bath. A further stirring step of 1h was also performed. NCs coated with new ligands were precipitated using an excess of hexane, then they were dissolved in water, forming a clear solution. A sonication step of 30 min gave a better colloidal dispersion. After 0.2 µm syringe filtration, excess of cys-PIMA-PEG was removed *via* 5 cycles of dilution/concentration using an amicon centrifuge filter (100 kDa, 3000 rpm, 12 min) in which the NCs solution diluted in 15 mL of water was concentrated down to circa 250-300 µL. The second cleaning cycle was performed using 15 mL of NaOH 0.1 M, while all the other cycles were performed with DI water. Selenium and zinc content in the final ZnSe-cys-PIMA-PEG solution was determined *via* elemental analysis.

ZnS and $CuFeS_2$ were transferred in water following a similar protocol. In the case of $CuFeS_2$ NCs, no $Zn(NO_3)_2$ was added.

The ZnSe ligand exchange with SH-PEG-OCH_3 was performed following a similar approach. In a 40 mL vial, 1 mL of ZnSe toluene solution (5.6 nm, 13µM) was precipitated using a slight excess of methanol, then it was centrifuged and collected as a dry pellet. In a different vial, 180 mg of SH-PEG2000-OCH_3 (2000 g/mol, Rapp Polymere, containing 0.09 mmol of –SH group, corresponding to about 73 SH/nm^2) was dissolved in 3 mL of ethanol and mixed with 100 mg of $Zn(NO_3)_2$•$6H_2O$. The ligand solution was then mixed with the NC's pellet. The NCs were quickly dissolved in ethanol and the mixture was further heated to 55 °C for 30 min to ensure a better ligand exchange. The NCs were then precipitated by adding an excess of hexane and dissolving the NC pellet in water, yielding a clear homogenous solution. After 0.2 µm of syringe filtration, an excess of SH-PEG-OCH_3 molecules were removed *via* 3 cycles of dilution/concentration using an amicon centrifuge filter (100 kDa, 3000 rpm, 12 min) in which the NC solution diluted in 15 mL of water was concentrated down to circa 250-300 µL. The selenium and zinc content in the final ZnSe- SH-PEG-OCH_3 dispersion was determined *via* elemental analysis.

S7 Stability of ZnSe coated with mono-dentate and multi-dentate ligand

ZnSe NCs coated with SH-PEG-OCH_3 and Cys-PIMA-PEG were subjected to a cation exchange reaction involving copper-64 (^{64}Cu, see section S11), and then purified *via* amicon centrifugation following the scheme reported in **Figure S4a**. The activity of all the parts was measured and reported as a percentage of the total activity.

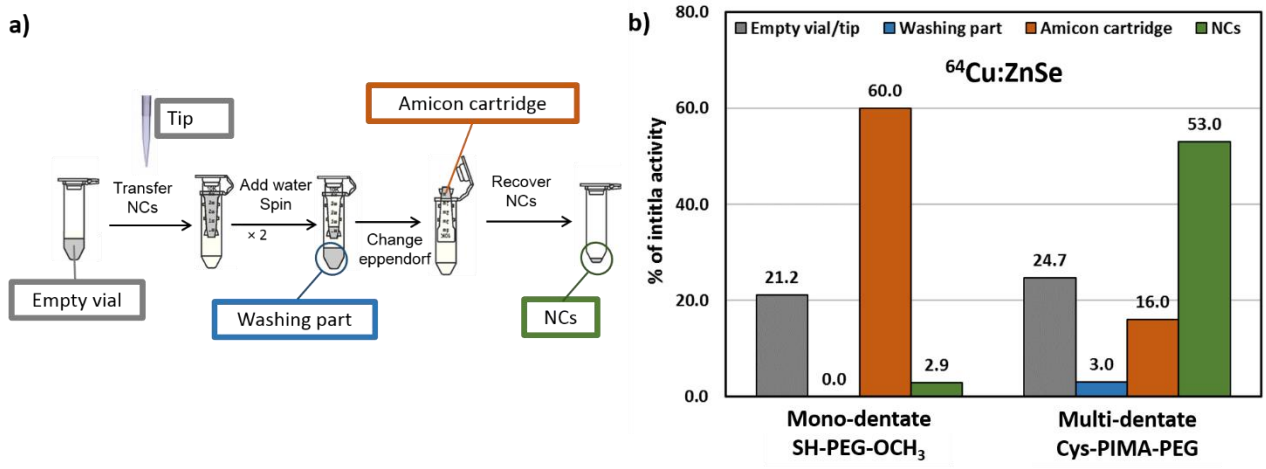


Figure S4 a) Scheme of the amicon purification steps upon the cation exchange reaction; b) The percentage of the total activity measured for each plastic part involved in the purification steps

S8 Cation exchange reaction

In a typical reaction, 10 µL of 2-15 µM NCs with an anion concentration (sulfur or selenium) ranging from 0.024 M to 0.049 M were diluted with 100 µL of MES buffer (50mM, pH 5.5) or water and mixed with a proper amount of $CuCl_2$ 0.021 M solution in HCl 0.01 M and excess of AA 0.1 M. The amount of $CuCl_2$ was properly tuned in order to obtain the desired final Cu/S or Se ratio. The AA content was determined taking into account a five-fold molar excess with respect to the molar amount of $CuCl_2$. For example, in the case of a total cation exchange reaction (Cu/Se or Cu/S ratio 1.8) with [S or Se] of 0.05 M, the amount of $CuCl_2$ solution was 42.9 µL and AA 42 µL. The mixture was incubated at 60°C or RT for 60 min. The reaction mixture was transferred into a 100 kDa cut-off amicon centrifuge filter (0.5 mL volume) and diluted up to 450 µL with water. Three cycles of amicon centrifugation (4000 rcf., 10 min) were performed in order to remove the released zinc and any excess ascorbic acid, collecting a final volume of circa 30-35 µL.

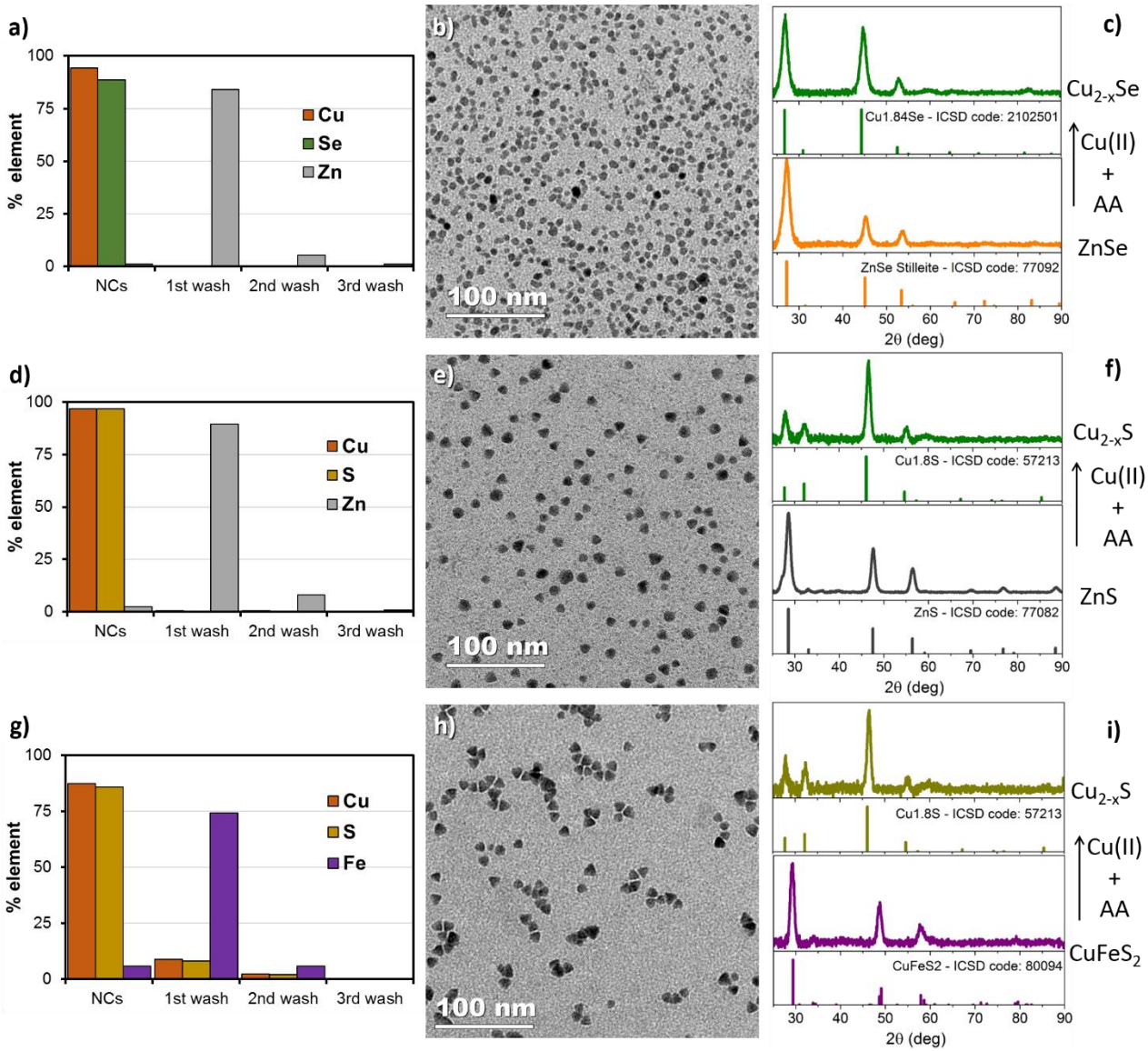


Figure S5 a) Elemental analysis of the NCs and washing steps after the amicon purification of ZnSe, which was subjected to a cation exchange reaction (ratio Cu/Se 1.8); b) TEM picture of ZnSe NCs after the cation exchange; c) XRD pattern of the NCs before (ZnSe) and after ($Cu_{2-x}Se$) cation exchange reaction. d) Elemental analysis of the NCs and washing steps after the amicon purification of ZnS, which was subjected to a cation exchange reaction (ratio Cu/S 1.8); e) TEM picture of ZnS NCs after the cation exchange; f) XRD pattern of the NCs before (ZnS) and after ($Cu_{2-x}S$) cation exchange reaction. g) Elemental analysis of the NCs and washing steps after the amicon purification of $CuFeS_2$, which were subjected to a cation exchange reaction (ratio Cu/S 1.8); h) TEM picture of $CuFeS_2$ NCs after the cation exchange; i) XRD pattern of the NCs before ($CuFeS_2$) and after ($Cu_{2-x}S$) cation exchange reaction.

		NPs (g)	Activity (GBq)	RCY (unit)	Specific activity (TBq/g)
CHELATOR	Dual-Function probe for PET and near-infrared fluorescence imaging of tumor vasculature[5]	5.35×10^{-5}	0.037	1	*0.692*
	microPET-based biodistribution of quantum dots in living mice[6]	7.46×10^{-5}	0.044	1	*0.595*
	Synthesis of ^{64}Cu-labeled magnetic nanoparticles for multimodal imaging[7]	1.56×10^{-3}	0.008	1	*0.005*
	In vivo evaluation of ^{64}Cu-labeled magnetic nanoparticles as a dual modality PET/MT imaging agent[8]	5.00×10^{-3}	0.111	1	*0.022*
	Synthesis, colloidal stability and ^{64}Cu labeling of iron oxide nanoparticles bearing different macrocyclic ligands[9]	1.00×10^{-3}	5.03×10^{-5}	1	*5.0×10^{-5}*
	Affibody modified and radiolabeled golf-iron oxide hetero-nanostructures for tumor PET, optical and MT imaging[10]				*0.11×10^{-3} Value in the paper*
CHELATOR FREE	A chelator-free multifunctional ^{64}Cu-CuS nanoparticle platform for simultaneous micro-PET/CT imaging and photothermal ablation therpay[11]	9.56×10^{-5}	0.037	1	*0.387*
	PET and NIR optical imaging using self-illuminating ^{64}Cu-doped chelator free gold nanoclusters[12]	9.85×10^{-5}	0.222	1	*0.225*
	Self-illuminating ^{64}Cu-doped CdSe/ZnS nanocrystals for in vivo tumor imaging[13]	1.00×10^{-3}	7.4×10^{-3}		*7×10^{-3} Value in the paper (200μCi/mg)*
	Chelator-free ^{64}Cu-integrated gold nanomaterials for positron emission tomography imaging guided photothermal cancer therapy[14]	1.5×10^{-4}	5.6×10^{-3}	1	*0.037*
	Copper-64 alloyed gold nanoparticles for cancer imaging: improved radiolabel stability and diagnostic accuracy[15]	8.3×10^{-3}	0.750	1	*0.090*
	Post-synthesis incorporation of ^{64}Cu in CuS nanocrystals to radiolabel photothermal probes: a feasible approach for clinics[16]	1.02×10^{-4}	0.037	0.5	*0.181*
	Industrial-scale synthesis of intrinsically radiolabeled ^{64}CuS nanoparticles for use in positron emission tomography (PET) imaging of cancer[17]	9.56×10^{-3}	9.99	1	*1.046*
	$FeSe_2$-decorated Bi_2Se_3 nanosheets fabricated via cation exchange for chelator-free ^{64}Cu-labeling and multimodal image-guided photothermal-radiation therapy[18]	3.00×10^{-5}	0.150	1	*5*
	^{64}Cu-doped PdCu@Au tripods: a multifunctional nanomaterial for positron emission tomography and image-guided photothermal cancer treatment	1.82×10^{-3}	1.28	1	*0.704*
	Mouse positron emission tomography study of the biodistribution of gold nanoparticles with	4.7×10^{-5}	2.028	0.5	*21.57*

	different surface coatings using embedded copper-64[19]				
Our protocol	ZnSe-cys-PIMA-PEG	8.66×10^{-7}	0.037	1	*43*
	$CuFeS_2$-cys-PIMA-PEG	5.0×10^{-7}	0.037	1	*74*
	ZnS-cys-PIMA-PEG	5.85×10^{-7}	0.037	1	*63*

Table S2: A summary of specific activities calculated for the different published procedures and grouped by approaches used to introduce the ^{64}Cu to the NCs.

S9 Photothermal properties

Complete cation exchange reactions were carried out in a 96-multi well plate, and each reaction occurred in a final volume of 200 µL. Reference solutions (not-exchanged) were prepared using the same quantity of NCs and the same final volume. The Cu/Se or S ratio was 1.8. The ascorbic acid (AA) was 0.1 M, and $CuCl_2$ was 0.0216 M (**Table S1** summarizes main chemicals amounts). The reactions were incubated at RT for 60 min, then, irradiated with a NIR laser (RTLMDL-808−5W, Roithner Laser Technik, 808 nm, 2 W/cm^2) for 3 min. The temperature was monitored using a thermocamera (Fluke Ti200)

	Vol NCs (µL)	Conc Se or S (M)	Vol $CuCl_2$ (µL)	Vol AA (µL)	Vol water (µL)
ZnSe	15	0.049	61	30	94
ZnS	15	0.089	111	50	24
$CuFeS_2$	15	0.028	25	15	145

Table S1

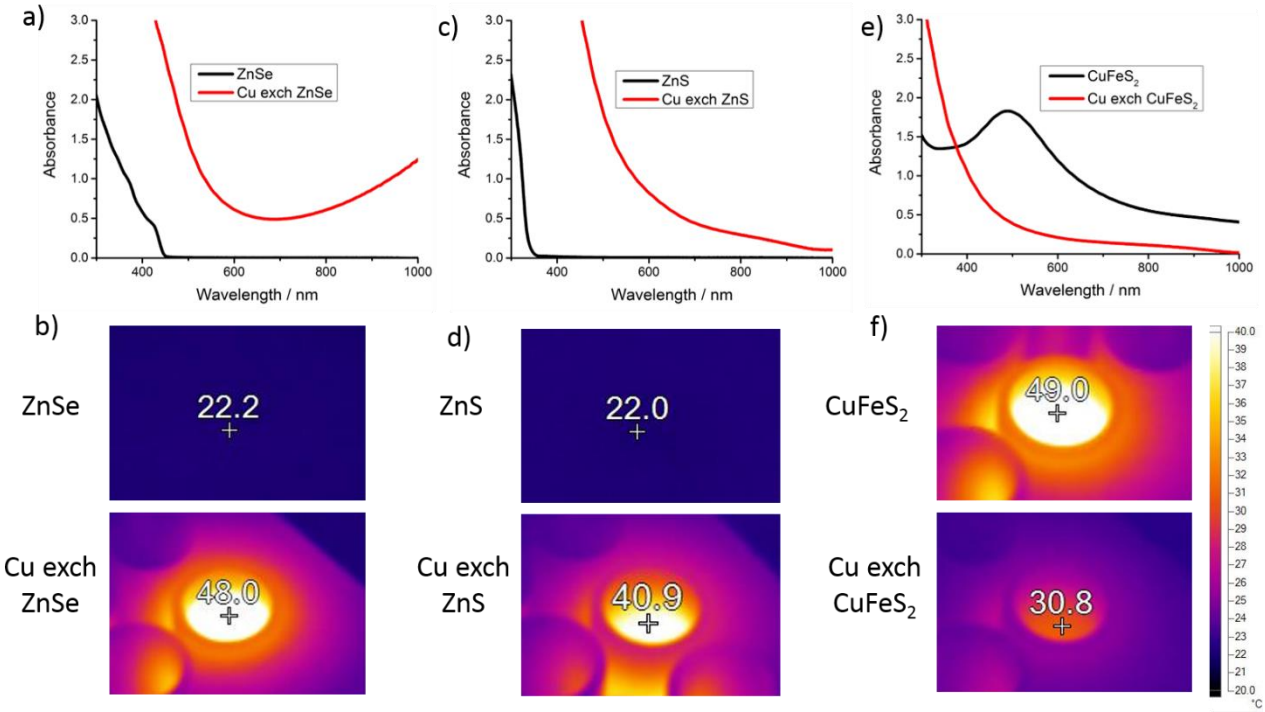


Figure S6 Absorption spectra before (black line) and after (red line) the Cu exchange on water soluble NCs ("a" ZnSe, "c" ZnS and "e" $CuFeS_2$). Photothermal picture of NCs before and after Cu exchange upon 3 min irradiation with a 808 nm laser at 2 W/cm^2 ("b" ZnSe, "d" ZnS and "f" $CuFeS_2$).

S10 Stability test

Stability test in pure water

The NCs that were fully exchanged with Cu ions in CE reaction, were then incubated in pure water (200 µL) at 37 °C for 24 h. One cycle of amicon centrifuge filter (0.5 mL) was applied in order to separate the NCs and the released copper that passed through the membrane. Copper, selenium and sulfur content were determined *via* ICP.

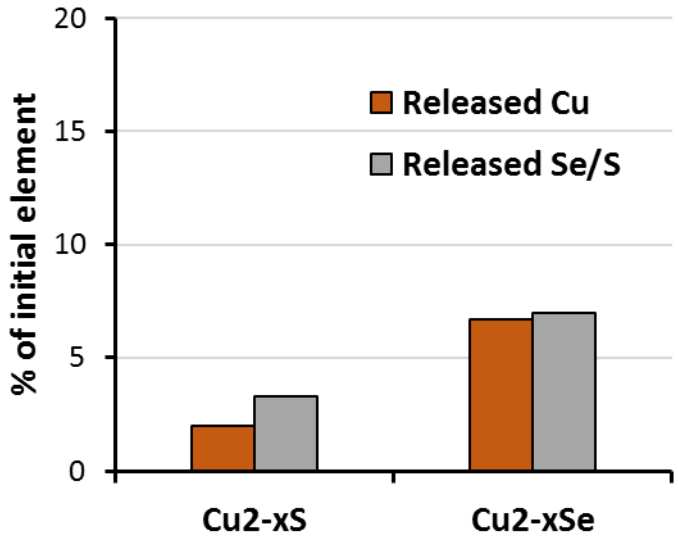


Figure S7 Copper released on NCs that had been subjected to a total cation exchange upon 24 h of incubation in water at 37 °C.

Stability test in human serum

Stability test in human serum (from male AB clotted whole bood, Sigma Aldrich) was performed by diluting a solution of NCs after cation exchange reaction with 200 µL of human serum, previously filtered with 0.45 µm syringe filter. Solution was incubated at 37 °C for 24 h. A reference test with pure human serum mixed with $CuCl_2$, previously neutralized in PBS 1X, was also performed. The mixture was then transferred in an amicon centrifugation device (0.5 mL, 100 kDa), diluted with 150 µL of EDTA solution (0.1 M, pH 5.9) and concentrated down to 100 µL at 4000 rcf. Upper part was then diluted with 200 µL of water and concentrated down to 100-50 µL. Elemental analysis was performed on both NCs solution and filtered solution and results reported as percentage of recovered copper (**Figure S8**).

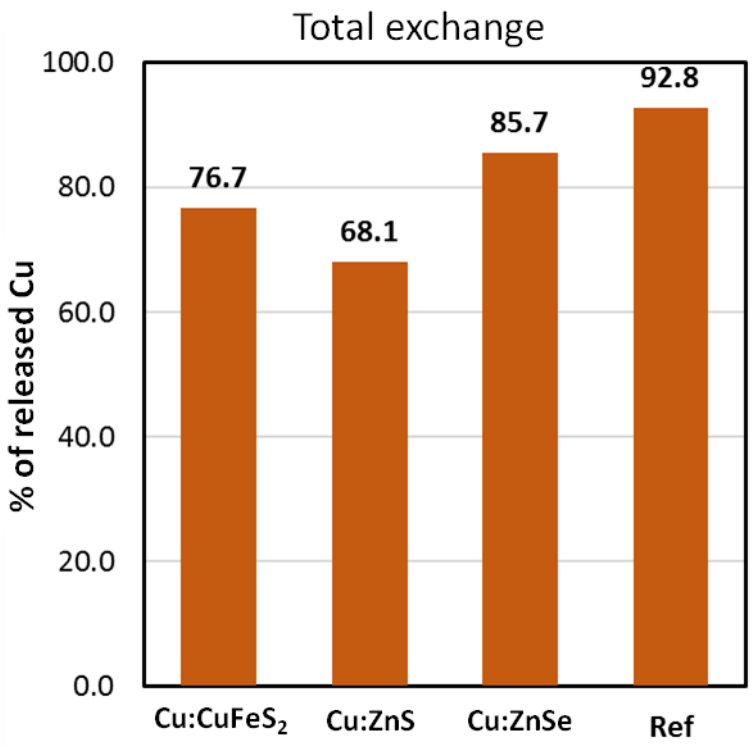


Figure S8 Copper leakage in human serum after 24 h of incubation for Cu:$CuFeS_2$, Cu:ZnS, Cu:ZnSe NCs upon a copper exchange with a Cu/S or Cu/Se ratio of 1.8 (b). A reference test (named ref) of $CuCl_2$ solutions was also incubated with human serum and treated as the other samples before elemental analysis is also reported.

S11 Radiolabeling reactions

Radiolabeling reactions carried out at 50°C was performed using $^{64}CuCl_2$. The production of ^{64}Cu was performed at a Cyclone®18/9 (Helmholtz-Zentrum Dresden-Rossendorf). For the $^{64}Ni(p,n)^{64}Cu$ nuclear reaction, 10 MeV protons with a beam current of 12 µA for 150 min were used. The yields of the nuclear reaction $^{64}Ni(p,n)^{64}Cu$ were 3.6–5.2 GBq [at the end of bombardment (EOB)] with molar activities of 50–100 GBq µmol−1 Cu diluted in HCl (10 mM).[20]A typical stock solution has an activity concentration of 500 MBq in 200 µL with a specific activity of 50 GBq/µmol Cu. The pH of the solution was adjusted to 5-6 using 4 M NaOH before the reaction. The moles of total copper found in the solution can be calculated from the activity of a certain volume of ^{64}Cu solution and the molar activity of the ^{64}Cu solution, following **Eq. S5.** In one single radiolabeling reaction of 37 MBq, taking into account the specific activity of 50 GBq/µmol $^{64}CuCl_2$, moles of total copper would be 7.4×10^{-10}.

$$moles\ of\ total\ Cu\ = \frac{Activity\ (MBq) \times\ 10^{-3}}{Molar\ activity\ (GBq/\mu mol)} \times\ 10^{-6} \qquad \text{Eq. S5}$$

Moles of ^{64}Cu can be calculated following the equation (**Eq. S6**)

$$moles\ of\ {}^{64}Cu\ = \frac{A_{Bq}(s^{-1})}{N_A(mol^{-1})} \times \frac{t_{1/2}(s)}{\ln(2)} \qquad \text{Eq. S6}$$

where A_{Bq} is the activity in Bq of the solution, N_A is the Avogadro's number, and $t_{1/2}$ is the half life of ^{64}Cu (12.7 h). In the case of the 37 MBq solution, the moles of ^{64}Cu would be 4.05×10^{-12}. This means that the total copper / hot copper ratio is 182.

The amount of NCs per reaction was calculated taking into account the copper associated with 37 MBq of activity and a ratio Cu:S or Cu:Se of 13 %. The concentration of the NCs was calculated as reported in section "S4 Determination of nanocrystal concentration"

$$Vol\ NCs\ (\mu L) = \frac{moles\ of\ total\ Cu}{ratio\ Cu{:}S\ or\ Se(unit)} / conc\ S\ or\ Se\ (mol/L) \times\ 10^{6} \qquad \text{Eq. S7}$$

The specific activity of NCs for each reaction was calculated following **Eq. S1**, taking into account the mass of the NCs (from ICP-OES results), the activity used in a single radiolabeling reaction (**Table S2**) and a RCY of 1.

	ZnSe	ZnS	$CuFeS_2$

	MW 354700 g/mol	MW 1288700 g/mol	MW 591400 g/mol
Activity (MBq)	37	37	37
Total Cu added (mol)	7.4×10^{-10}	7.4×10^{-10}	7.4×10^{-10}
Ratio Cu/S or Cu/Se	13 %	13 %	13 %
Conc element (mM)	Se 0.6 Zn 0.6	Zn 0.6 S 0.6	Cu 0.15 Fe 0.15 S 0.3
Vol NCs (µL)	10	10	18
Mass NCs (g)	8.66×10^{-7}	5.85×10^{-7}	5.00×10^{-7}
moles NCs	2.44×10^{-12}	4.54×10^{-13}	8.45×10^{-13}
Conc NCs (mol/L)	2.44×10^{-7}	4.54×10^{-8}	4.70×10^{-8}
Specific activity (TBq/g)	43	63	74

Table S2 Characteristics and quantity of all NCs employed in each radiolabeling reaction

In a 1.5 mL Eppendorf vial were added 100 µL of MES buffer (0.1M, pH 5.6), 10 µL of ascorbic acid (0.1 M), a proper amount of NCs (see **Table S2** for details) and ^{64}Cu solution (37 MBq). The solutions were incubated at 50 °C for 1h. Radiolabeling process was monitored by radio-thin layer chromatography (radio-TLC) using instant TLC (iTLC-SG) plates, which were purchased from Agilent Technologies, and 0.1 M EDTA as a developing agent. Subsequently, the plates were read by a radioluminography laser scanner BAS-1800II (Raytest). Data analysis was performed using Aida image analyzer version 4.0. Data have been summarized in **Figures S9** (radiolabeling: a) – c) and challenging the ^{64}Cu-labeled NCs with 0.1 M EDTA for 20 min d) – f)).

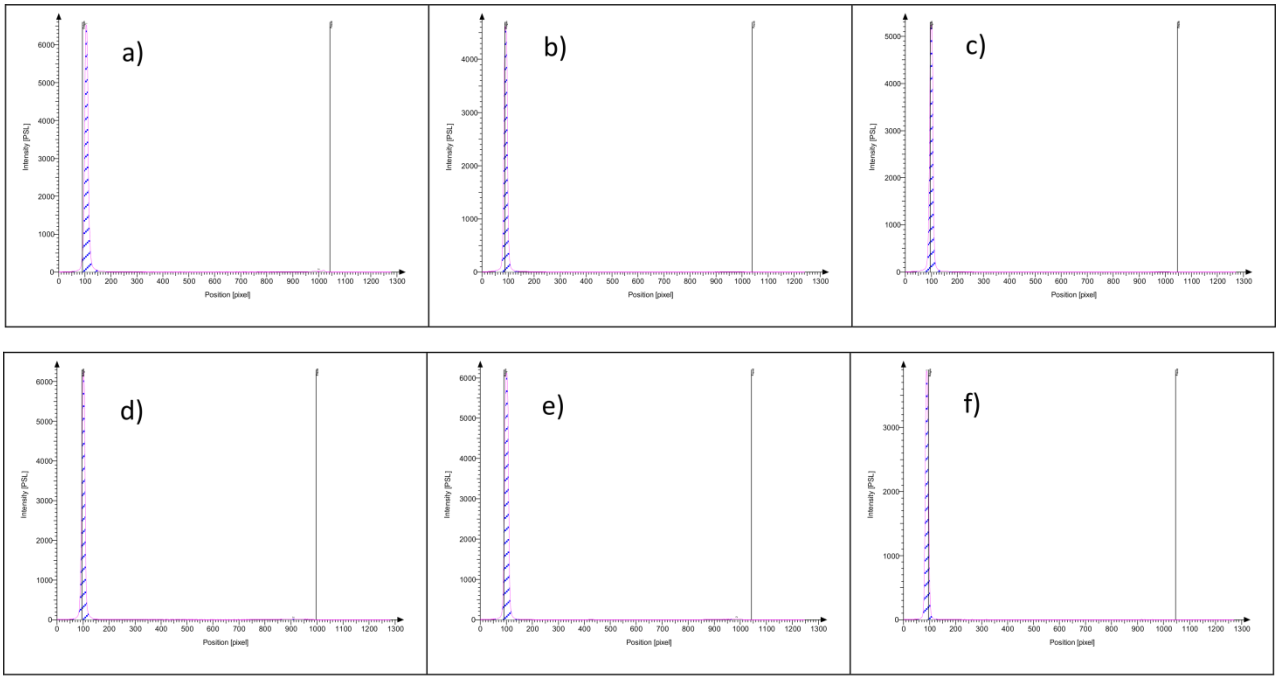


Figure S9 Results from radio-iTLC integration of a) 64Cu:ZnS, b) 64Cu:ZnSe, c)^{64}Cu:$CuFeS_2$, radiolabeling at 50 °C for 1 h (RCY > 99%); d) ^{64}Cu:ZnS (1.5% ^{64}Cu release), e) ^{64}Cu:ZnSe (1% 64Cu release), f)^{64}Cu:$CuFeS_2$ (< 1% 64Cu release), challenging ^{64}Cu-NCs with 0.1 M EDTA for 20 min.

NCs were purified via amicon centrifuge filtration (**Figure S4a**), and the activity of all plastic parts and materials involved in the separation procedure were analyzed using an activity counter (ISOMED 1000, MED Nuklear-Medizintechnik Dresden GmbH). The results are reported in **Figure S9.** The purified NCs were dissolved in 200 µL of human serum for a stability test at 37 °C. Radio-TLC was performed after 1 h and 24 h, using EDTA 0.1 M as mobile phase (**Figure S11**).

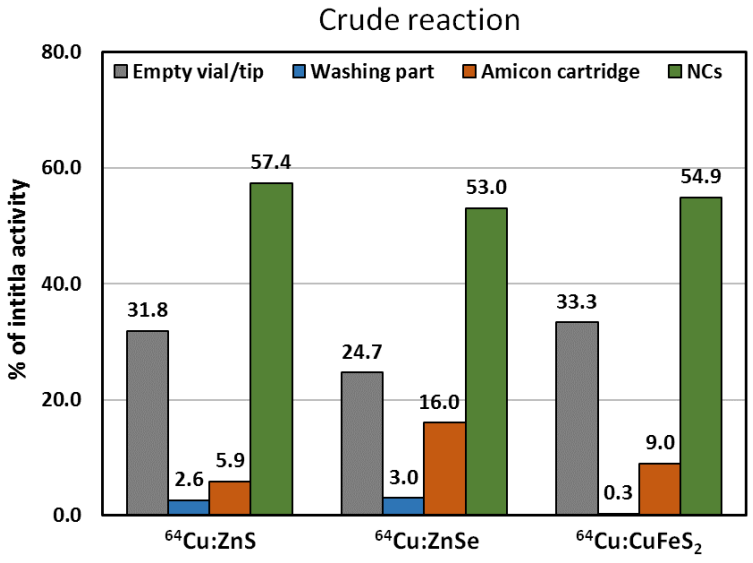


Figure S9 Percentage of initial activity recovered upon amicon centrifugation of the reaction mixture.

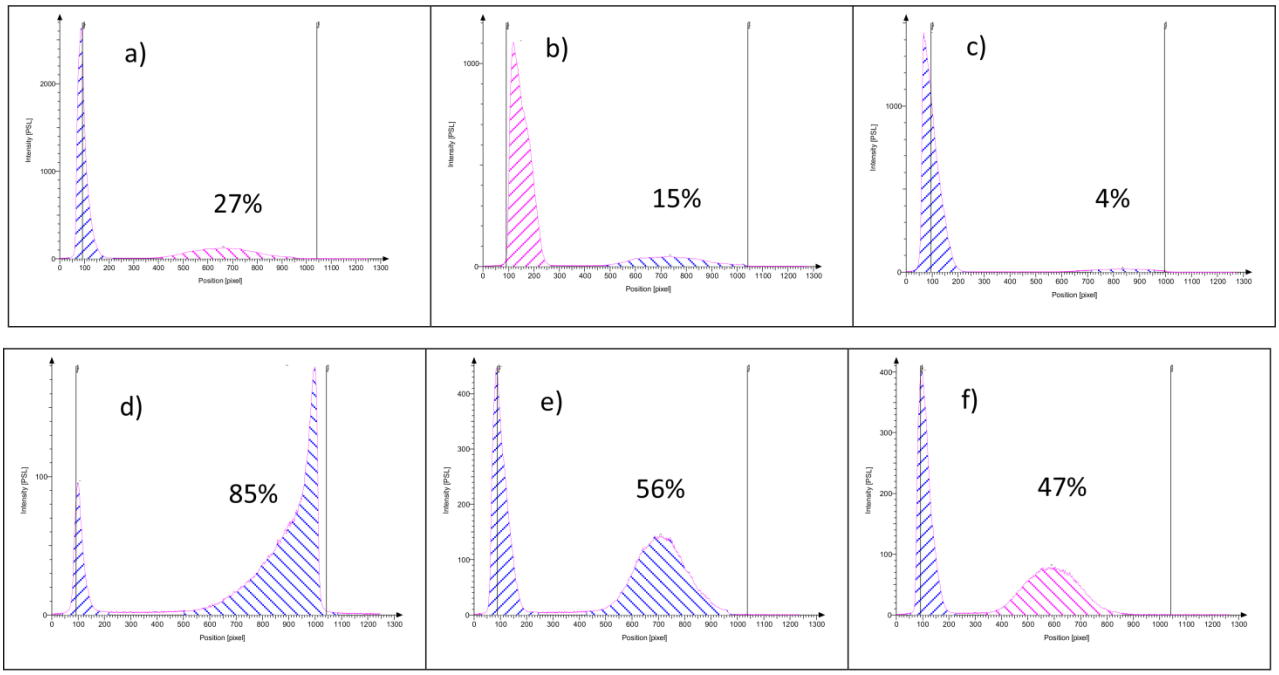


Figure S10 Results from radio-iTLC integration of ^{64}Cu released upon incubation NCs in human serum at 37 °C for 1h: a) ^{64}Cu:ZnS, b) ^{64}Cu:ZnSe, c)^{64}Cu:$CuFeS_2$ and 24 h: d) ^{64}Cu:ZnS, e) ^{64}Cu:ZnSe, f)^{64}Cu:$CuFeS_2$.

<u>Radiolabeling reaction at different amount of nanocrystals</u>

Radiolabeling reactions at different nanocrystal concentrations and carried out at 37°C were performed following a similar protocol as before. Copper-64 reagent was a commercially available aqueous solution of $^{64}CuCl_2$ dissolved in HCl 0.1M with a specific activity of 140 GBq/µmol (Cuprymina, ACOM s.p.a.). The activity per reaction was set to 18.5 MBq (0.5 mCi). Based on **Eq. S5**, taking into account 18.5 MBq as the activity and 140 GBq/µmol as the specific activity of the ^{64}Cu solution, amount of Cu per reaction was calculated to be 1.32×10^{-10}. The concentration of NCs was calculated based on **Eq. S4**.

	Reaction 1	**Reaction 2**	**Reaction 3**	**Reaction 4**	**Reaction 5**
Conc S/Se stock (mM)	0.2	0.4	0.8	3.3	6.5
Conc ZnSe NC (mol/L)	8.1×10^{-8}	1.6×10^{-7}	3.3×10^{-7}	1.3×10^{-6}	2.6×10^{-6}

Conc ZnS NC(mol/L)	1.5×10^{-8}	3.0×10^{-8}	6.0×10^{-8}	2.5×10^{-7}	4.9×10^{-7}
Conc $CuFeS_2$ NC (mol/L)	3.1×10^{-8}	6.2×10^{-8}	1.2×10^{-7}	5.1×10^{-7}	1.0×10^{-6}

Table S3 Summary of concentration of NC stock solutions employed in radiolabeling reactions when changing the NC amount

Expected specific activity was calculated (**Eq. S1**) taking into account the mass of the NCs (**Eq. S8**) and the activity that was used in a single radiolabeling reaction (**Table S4**, **Table S5** and **Table S6**)

$$mass\ NC\ (g) = Conc\ NCs\ (mol/L) \times Vol\ NCs\ (\mu L) \times 10^{-6} \times MW\ NCs\ (g/mol) \quad \text{Eq. S8}$$

ZnSe 354700 g/mol	**Conc NCs (mol/L)**	**Vol NCs (µL)**	**Ratio Cu/Se (%)**	**mol NCs**	**mass NCs (g)**	**Activity (MBq)**	**Specific activity (TBq/g)**
Reaction 1	8.1×10^{-8}	10	6.6 %	8.1×10^{-13}	2.9×10^{-7}	18.5	63
Reaction 2	1.6×10^{-7}	10	3.3 %	1.6×10^{-12}	5.8×10^{-7}	18.5	32
Reaction 3	3.3×10^{-7}	10	1.7 %	3.3×10^{-12}	1.2×10^{-6}	18.5	15
Reaction 4	1.3×10^{-6}	10	0.4 %	1.3×10^{-11}	4.8×10^{-6}	18.5	3.9
Reaction 5	2.6×10^{-6}	10	0.2 %	2.6×10^{-11}	9.4×10^{-6}	18.5	2.0

Table S4 Volume, concentration, moles and mass of ZnSe, Cu/Se ratio and specific activity of the radiolabeling reactions upon variation of NCs amount.

ZnS 1288700 g/mol	**Conc NCs (mol/L)**	**Vol NCs (µL)**	**Ratio Cu/S (%)**	**mol NCs**	**mass NC (g)**	**Activity (MBq)**	**Specific activity (TBq/g)**
Reaction 1	1.5×10^{-8}	10	6.6 %	1.5×10^{-13}	1.9×10^{-7}	18.5	97
Reaction 2	3.0×10^{-8}	10	3.3 %	3.0×10^{-13}	3.9×10^{-7}	18.5	47
Reaction 3	6.0×10^{-8}	10	1.7 %	6.0×10^{-13}	7.8×10^{-7}	18.5	23
Reaction 4	2.5×10^{-7}	10	0.4 %	2.5×10^{-12}	3.2×10^{-6}	18.5	5.8
Reaction 5	4.9×10^{-7}	10	0.2 %	4.9×10^{-12}	6.3×10^{-6}	18.5	2.9

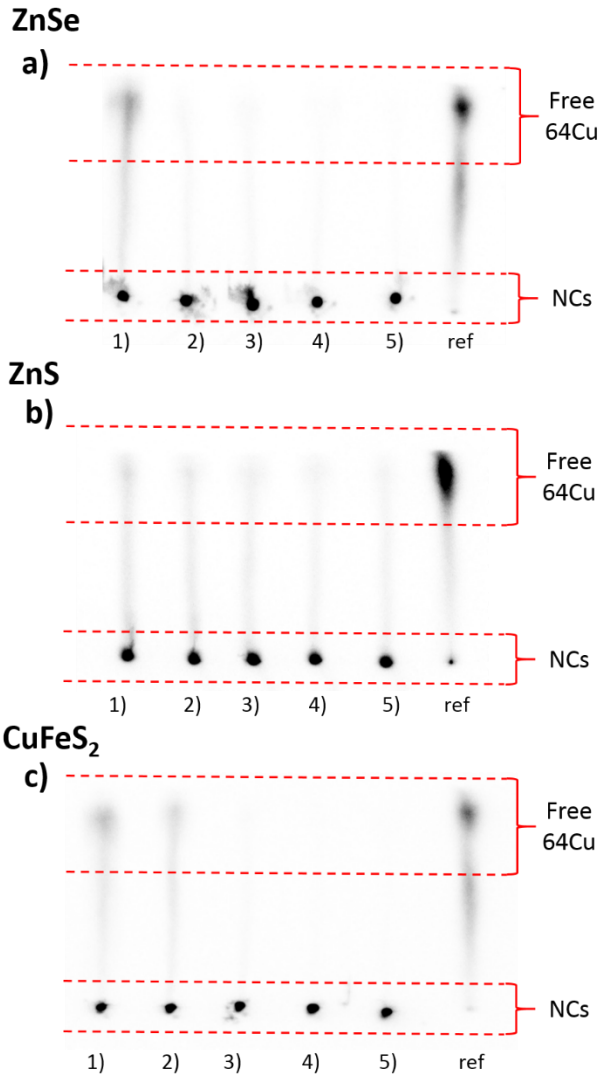
Table S5 Volume, concentration, moles and mass of ZnS, Cu/S ratio and specific activity of the radiolabeling reactions upon variation of NCs amount

$CuFeS_2$ 591400 g/mol	**Conc NCs (mol/L)**	**Vol NCs (µL)**	**Ratio Cu/S (%)**	**mol NCs**	**mass NC (g)**	**Activity (MBq)**	**Specific activity**

							(TBq/g)
Reaction 1	3.1×10^{-8}	10	6.6 %	3.1×10^{-13}	1.8×10^{-7}	18.5	103
Reaction 2	6.2×10^{-8}	10	3.3 %	6.2×10^{-13}	3.7×10^{-7}	18.5	50
Reaction 3	1.2×10^{-7}	10	1.7 %	1.2×10^{-12}	7.3×10^{-7}	18.5	25
Reaction 4	5.1×10^{-7}	10	0.4 %	5.1×10^{-12}	3.0×10^{-6}	18.5	6.1
Reaction 5	1.0×10^{-6}	10	0.2 %	1.0×10^{-11}	6.0×10^{-6}	18.5	3.1

Table S6 Volume, concentration, moles and mass of $CuFeS_2$, Cu/S ratio and specific activity of the radiolabeling reactions upon variation of NCs .

In 1.5 mL Eppendorf vial, were added 150 µL of MES buffer (0.3 M, pH 5.6), 5 µL of AA 0.1 M and 10 µL of NCs (see **Table S4**, **Table S5** and **Table S6** for concentration). Upon the addition of a ^{64}Cu copper chloride solution (31.3 µL, corresponding to 18.5 MBq), the reactions were incubated at 37 °C for 1 h. The radiolabeling process was monitored *via* radio-thin layer chromatography (radio-TLC) using instant TLC (iTLC-SG, Agilent Technologies) and EDTA 0.1 M pH 5.9 as a developing agent. The TLC plates were read by a radioluminography laser scanner (Fujifilm FLA-9000 Starion). The images were analyzed using a ImageJ software (version 1.8.0). Radiochemical yield and radiochemical purity were determined integrating the activity on the deposition point (NCs) and on the front of the solvent (free ^{64}Cu). The results are gathered in **Figure** .



	Deposition point (NCs)	Front (free 64Cu)
Reaction 1)	75 %	25 %
Reaction 2)	100 %	
Reaction 3)	100 %	
Reaction 4)	100 %	
Reaction 5)	100 %	
Ref 64Cu	2 %	98 %

	Deposition point (NCs)	Front (free 64Cu)
Reaction 1)	80 %	20 %
Reaction 2)	96 %	4 %
Reaction 3)	97 %	3 %
Reaction 4)	99.5 %	0.5 %
Reaction 5)	99 %	1 %
Ref 64Cu	4.5 %	95.5 %

	Deposition point (NCs)	Front (free 64Cu)
Reaction 1)	69 %	31 %
Reaction 2)	91 %	9 %
Reaction 3)	100 %	
Reaction 4)	100 %	
Reaction 5)	100 %	
Ref 64Cu	2 %	98 %

Figure S12 Radio-TLC and integration of ^{64}Cu:ZnSe (a), ^{64}Cu:ZnS(b) and ^{64}Cu:$CuFeS_2$(c) of the reaction mixture.

Reaction mixture solution was purified following the amicon filtration procedure that has been reported for other reactions (**Figure S4**a). Activity of the Eppendorf vial, washing part and NCs fractions was measured using an automatic gamma counter (Wizard 1470; PerkinElmer, MA, USA). NCs fraction was diluted with 200 µL of human serum and incubated at 37°C for 24 h. Copper-64 released was quantified via radio-TLC using iTLC-SG as a substrate and EDTA as a developing agent.

S12 In vitro cellular study.

For *in vitro* proof of concept experiments to demonstrate the therapeutical efficacy of our radiolabeled NCs, both human glioblastoma U87 (ATCC, UK) and epidermoid carcinoma A431 (ATCC, UK) cell lines were used. Cells were cultured in Dulbecco's modified Eagle medium (MEME, Gibco, UK) supplemented with 10 % inactivated fetal bovine serum (FBS), 1 % penicillin streptomycin (PS) and 1 % glutamine at 37°C in 95 % humidity and 5% CO_2. Cells were split every 3-4 days before they reached 80% confluence. The cellular experiments were performed in Eppendorf tubes containing cell pellets (2 million cells each), For the cells-only control studies, the cells pellet was incubated with 50 µL PBS for 2h in the incubator at 37°C with 95% humidity and 5% CO_2. For the non-laser controls, a solution of $^{64}CuCl_2$, 37 MBq (110 µL) (with a pH previously adjusted at pH 6) was added to the cell pellet, while for ^{64}Cu:$CuFeS_2$, the concentrated NC (37 MBq, 50 µL, 230 pM) were added to the pellet. These vials were incubated on the heating block at 37°C for a total of 2h.

For the laser irradiation experiment, the cells pellets were incubated with the above-mentioned radioactivity and the cap of the Eppendorf vial was replaced with a paraffin film. The temperature probe was inserted into the vial, just touching the surface of the solution. Irradiation was performed using the laser setup (power 1.7 W/cm^2 for 13 min) and the temperature rise in the solution was monitored. The solutions were allowed to come to room temperature and the laser cycle was repeated for three times. The cell pellets were incubated with radioactivity for a total of 2h, including the laser irradiation time.

After 2h, the media was removed from the vial and the pellet was washed with PBS. The residual activity in the cell pellet was counted using a dose calibrator and the percentage of radioactivity uptake was calculated by dividing the radioactivity found in the cell pellet with the total radioactivity incubated on the cells. The cell pellets were then suspended in DMEM and a third of suspension was placed per well in a 12 well plate. After 16h, 24h and 48h, the PrestoBlue cell viability experiment was carried out according to the manufacturer's procedure. Briefly, at the end of the incubation period, the media containing NCs was removed, cells were washed with PBS and the PB reagent (Invitrogen) (10% PB in DMEM) was then added the wells containing cells and was incubated for an additional 60-120 min at 37 °C. The cell viability was determined at this stage by recording the absorbance for each well at 570 nm and 600 nm. The absorbance ratios for each well were normalized with respect to the absorbance ratio of the control cells wells, which were not treated with NPs.

S13 Cytotoxicity of NCs

The viability of the cells upon incubation with NCs was tested using a PrestoBlue assay (PB, ThermoFisher, Waltham, MA, USA) performed according to the reported protocol by the producer. Briefly, on the day prior to the experiment, U87 or A431 (6×10^4 cells/well for the 24 h incubation test and 8×10^4 cells/well for the 48 h incubation test) were seeded in a 24 multiwell plate and allowed to adhere to the well dish. On the day of experiment, the media was aspirated, cells were washed with PBS and the NCs (0-500 pM for $CuFeS_2$, 0-500 pM for ZnS and 0-1000 pM for ZnSe) were added to the media and were incubated for an additional 24 h or 48 h at 37 °C. At the end of the incubation period, the media containing NCs was removed, cells were washed with PBS and PrestoBlue cell viability was carried out as per the procedure explained above.

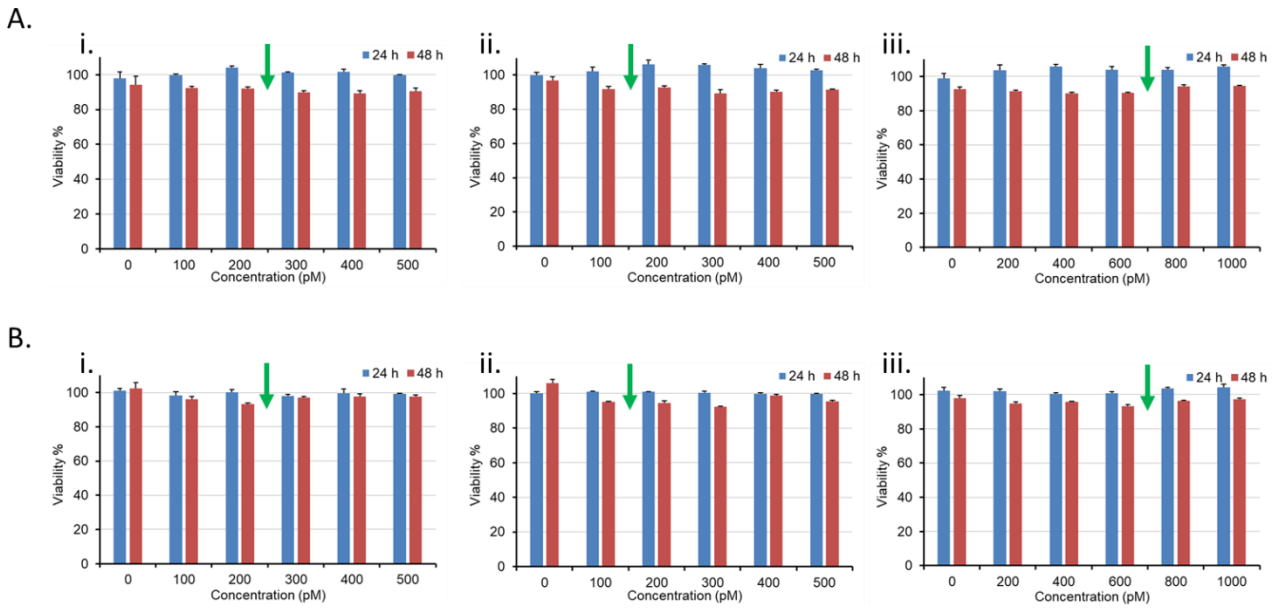


Figure S13 The cytotoxicity data obtained by presto blue viability test on A) A431 and B) U87 cancer cells. The indexes i, ii, iii stand for $CuFeS_2$, ZnS and ZnSe NCs respectively while the green arrows indicate the concentration of the NCs used for radiolabeling.

S14 References

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