Here, we exploit CE reactions to radiolabel ZnSe, ZnS and CuFeS$_2$ metal chalcogenide nanocrystals (NCs) with $^{64}$Cu. Our CE protocol requires one simple step, to mix the water-soluble NCs with a $^{64}$Cu solution, in the presence of vitamin C used to reduce Cu(II) to Cu(I). Given the
quantitative cation replacement on the NCs, a high radiochemical yield, up to 95%, is reached. Also, provided that there is no free $^{64}$Cu, no purification step is needed making the protocol easily translatable to the clinic. A unique aspect of our approach is the achievement of unprecedentedly high specific activity: by exploiting a volumetric cations exchange, our strategy enables to concentrate a large dose of $^{64}$Cu (18.5 MBq) in a small NC dose (0.4 μg), reaching a specific activity of 50 TBq/g. Finally, the characteristic dielectric resonance peak, still present for the radiolabeled $^{64}$Cu:CuFeS$_2$ NCs after the partial-CE reaction, enables the generation of heat under clinical laser exposure (1 W/cm$^2$). The synergic toxicity of photo-ablation and $^{64}$Cu ionization is here proven on glioblastoma and epidermoid carcinoma tumor cells, while no intrinsic cytotoxicity is seen from the NC dose employed for these dual experiments.

1. Introduction

Non-stoichiometric copper chalcogenide NCs are mainly known for their localized surface plasmonic resonance band, which peaks in the near infrared region (NIR).\cite{1} Thanks to this band, the energy of a NIR source can be transduced into macroscopic heat, making these materials promising heat mediators for photo-thermal therapy.\cite{2} The effectiveness of the photo-thermal behavior of both copper sulfide\cite{3} and copper selenide\cite{4} NCs has already been demonstrated in vitro and in vivo. A large variety of copper chalcogenides NCs can be prepared either by a direct synthesis approach (i.e. hot decomposition of copper and sulfur or selenium precursors) or by an indirect approach exploiting CE reactions,\cite{5} that is, by replacing pristine cations with copper ions on preformed NCs,\cite{6} making it possible to access a large variety of NC compositions that cannot be prepared by direct synthesis.\cite{7}
In clinic, radioisotopes are extensively used for both imaging and therapeutic applications. Depending on the imaging or therapeutic purpose, the radionuclide has to fulfill certain requirements in terms of the type of ionizing particles that are released and their decay time.\textsuperscript{[8]} \textsuperscript{64}Cu decays through $\beta^-$ particles (0.573 MeV, 39 \%) and electron capture (44 \%) emission, which are useful for radiotherapy applications\textsuperscript{[9]} but it can also produce a $\beta^+$ decay (0.655 MeV, 17 \%), therefore it is also suitable for positron emission tomography (PET) imaging.\textsuperscript{[10]} Furthermore, \textsuperscript{64}Cu is currently produced in many cyclotron facilities on a routine basis,\textsuperscript{[11]} and it is much more widely available than \textsuperscript{67}Cu, making the \textsuperscript{64}Cu an ideal candidate for preclinical research and for the development of radio-therapeutic agents.\textsuperscript{[12]}

Merging the properties of plasmonic photo-thermal NCs and radionuclides in one single nano-object can offer new combined approaches to cancer therapy.\textsuperscript{[13]} Up to date, \textsuperscript{64}Cu radiolabeled NCs were mostly used as imaging tools for PET.\textsuperscript{[14]} In some studies, theranostic applications have been pursued: the positron emission behavior of low dopant \textsuperscript{64}Cu was exploited for PET imaging, while the intrinsic material’s properties were used for therapeutic purposes (\textit{i.e.} for photo-thermal performances).\textsuperscript{[14b, 15]} Only one work has explored the synergistic effect of radiation and NIR (near infrared light) photo-thermal therapy of \textsuperscript{64}Cu-radiolabeled NCs.\textsuperscript{[16]} Zhou \textit{et al.}\textsuperscript{[16-17]} prepared \textsuperscript{64}Cu:CuS PEG-coated NCs by aqueous co-precipitation of copper (both “hot” \textsuperscript{64}Cu and “cold” Cu) and sulfur-containing salts, and administered intra-tumorally these radiolabeled NCs using a xenograft BT474 breast tumor mice model. They showed that the combination of both treatments was necessary to completely eradicate the tumor mass, as each individual therapy, either radiotherapy or heat therapy, was not sufficient. Moreover, this work suggests that \textsuperscript{64}Cu associated with NCs can greatly affect the retention time of radioisotopes,
enabling a longer persistence of the $^{64}$Cu radionuclide at the tumor site than when using the bare $^{64}$CuCl$_2$ solution.

Accumulating a high radionuclide dose at the tumor site, by increasing the specific activity of $^{64}$Cu:NC, is the key to maximize the radio-therapeutic efficacy. The specific or molar activity is defined as the amount of radioactivity per unit mass or mole of material. In other words, it represents the amount of radionuclides associated to a carrier (e.g. a nanoparticle, antibody, chelating ligand etc.). The use of radiolabeled NCs with a high specific activity would enable to administer a lower NC dose, thus minimizing cytotoxic side effects, without compromising the radionuclide dose that must be delivered for a more effective therapy.

NCs coupled with proper chelating agents that can bind $^{64}$Cu at their surface$^{[18]}$ are expected to be less nephrotoxic$^{[19]}$ than antibodies or proteins bearing a radionuclide.$^{[20]}$ These NCs, however, due to the limited number of chelating units per NC surface, have specific activity values around 0.7 TBq/g.$^{[21]}$ Moreover, the detachment of the radiometal from the NC surface should be taken into account when the radiolabeled NCs are exposed to the biological fluid.$^{[22]}$ The insertion of $^{64}$Cu ions inside the NCs has been proposed as an alternative method to produce stable radiolabeled NCs.$^{[23]}$ Radionuclides can be inserted into the NCs either during the NC synthesis$^{[15a, 24]}$ or in a post-synthesis step, following CE or intercalation reactions.$^{[14a, 25]}$ The direct incorporation of $^{64}$Cu into a NCs core during the synthesis has provided NC with a higher specific activity (2 TBq/g) than chelating methods.$^{[16]}$ However, no in situ synthesis has led to a quantitative insertion of $^{64}$Cu but only radiotracer doses for PET imaging were reported. CE or intercalation reactions are feasible for a quantitative replacement of cations, thus significantly increasing the specific activity of the radiolabeled NCs. Indeed, exchanging only 10% of the cations in a 6 nm spherical shaped zinc selenide (ZnSe) NC with a cubic sphalerite structure, using
37 MBq of a $^{64}$Cu solution (50 GBq/µmol) would produce a material with a specific activity of around 43 TBq/g, assuming that a quantitative cation exchange (QCE) process occurs.

Here, we demonstrate that performing a quantitative CE reaction is feasible using a $^{64}$CuCl$_2$ solution of high specific activity on water-dispersible chalcogenide-based NCs of different materials, including ZnS, CuFeS$_2$ and ZnSe. In this work, we show that, by using a constant amount of $^{64}$Cu on a µg dose of NCs, the specific activity can be varied in a wide range (from 2 to 100 TBq/g), especially designed for a radiotherapeutic use. The use of a proper surface coating was crucial to develop radiolabeling protocols that provide NCs with an optimal colloidal and radiolabeling stability upon a $^{64}$Cu exchange. Among the various ligands that were available, we selected a multi-anchoring cysteamine-containing polyethylene glycol (PEG) ligand, as it does not interfere with the CE reaction and, at the same time, it provides the NCs with high colloidal stability in aqueous buffer or serum solutions. Finally, we want to highlight the simplicity of the radiolabeling procedure herein reported, which involves a single step consisting in the mixing of the NCs with the $^{64}$CuCl$_2$ solution, in buffer at 37°C for 1 hour, with no further need of purification steps. Such simplicity makes is easily implementable in any clinical hospital where $^{64}$Cu is available.

2. Results and discussion

For our radiolabeling protocol, we selected Cd-free chalcogenides NCs at different compositions to minimize toxicity effects of the NCs, including spherical Zn-based chalcogenides (ZnS and ZnSe, with a diameter of 10 nm and 6 nm, respectively), and tetrahedral CuFeS$_2$ chalcopyrite NCs with an edge length of around 13 nm. These NCs were synthetized following non-hydrolytic hot decomposition protocols reported in the literature, with minor modifications (Figure S1, S2 of the electronic supporting information, ESI). The as-synthetized NCs, coated
with hydrophobic ligands (e.g. oleylamine and/or oleic acid), were transferred in water following a ligand exchange procedure. \cite{27} We synthesized (by adapting an already reported procedure) and characterized a multi-anchoring ligand made of poly-isobutylene maleic anhydride (PIMA), equipped with cysteamine (cys) units, which act as anchors for the NC surface and having amine-polyethylene glycol-methoxy-terminated (PEG) molecules as water soluble units (Figure 1a), (Figure S3, ESI for more details).\cite{28} Water-transferred NCs deposited from an aqueous solution on a transmission electron microscopy (TEM) grid formed a monolayer of well-separated NCs, indicating homogenous NC samples, with no presence of aggregates (Figure 1: c, f and i). Dynamic light scattering (DLS) measurements indicated average hydrodynamic sizes for each of the three NC samples in between 15 and 25 nm, with low polydispersity (PDI) index (Figure 1: d, g and l). Both data confirm the absence of aggregates in the water transferred NCs. The multi-dentate cys-PIMA-PEG provided also a stable polymer shell to the NCs, minimizing the yield loss during the NCs water transfer to less than 20% (only a small fraction of NC was lost during the washing steps on an amicon cartridge) in accordance with other studies in which multidentate ligands were reported.\cite{28-29}

A first set of CE reactions were carried out employing “cold” copper, which is a non-radioactive copper salt (CuCl$_2$). CuCl$_2$ 0.021M in HCl (0.01M) was chosen because it mimics the clinically available CuCl$_2$ solution. These CE reactions were based on an in situ reduction of Cu(II) in the presence of a mild reducing agent, namely ascorbic acid (AA); (Figure 1b for the reaction scheme) to Cu(I) in a MES buffer. Differently from Cu(II), Cu(I) is known to exchange with Zn ions in ZnSe and ZnS NCs.\cite{30} In a typical reaction, 10 µL of 2-15 µM NCs (corresponding to an anion concentration of sulfur or selenium in between 0.02 to 0.05 M) were diluted with 100 µL of a MES buffer (0.1 M, pH 5.5) or water and mixed with the proper amount of AA (0.1M). The
amount of CuCl\(_2\) was adjusted in order to vary the final Cu/S or Cu/Se ratio so that a partial (13%, Figure 1: e, h and m) or a total (100%, Figure S5) nominal Cu exchanged with the NCs cations was achieved.

Cu, Zn, Fe, S and Se elemental analyses (by means of inductively-coupled plasma optical emission spectrometry (ICP-OES) were performed on the pristine NCs, on the NCs fraction after CE reaction (the exchanged NC samples are indicated as Cu:ZnSe, Cu:ZnS and Cu:CuFeS\(_2\) NCs) and after a cleaning step (the reaction mixtures were subjected to three cycles of concentration/dilution on amicon centrifuge filter) to carefully separate the cation exchanged NCs (Cu:ZnSe, Cu:ZnS and Cu:CuFeS\(_2\)) from the eventually unbound Cu ions and from the cations freed in solution (Zn and Fe). After CE reaction, the copper amount was found entirely in the NC fractions and no Cu was present in the washing solutions, indicating a complete incorporation of Cu into the NCs (Figure 1, panels e-h for Cu:ZnSe, Cu:ZnS and panel m for Cu:CuFeS\(_2\) for an exchange at 13%). The replaced ions, Zn\(^{2+}\) in case of ZnSe and ZnS and the Fe\(^{3+}\) for CuFeS\(_2\), as expected, were found instead in the washing solutions (named Filtrate in Figure 1: e, h and m). The Cu:NCs did not show any variation in colloidal stability (Figure 1: d, g and l). The same trend was also observed when increasing the initial amount of Cu to have a nominal exchange of almost 100% (Figure S5). It is worth mentioning that, for the complete CE reaction, the amount of Cu per NC can be ideally exchanged to the stoichiometric ratio Cu/Se or Cu/S of 2:1. However, the Cu:Se and Cu:NCs would be easily oxidized to the copper deficient chalcogenides Cu\(_{2-x}\)Se or Cu\(_{2-x}\)S\(^{31}\), and there would be a concomitant release of Cu ions in the solution (data not shown). Therefore, for a quantitative cation exchange, we chose to perform a copper CE reaction at a final Cu/Se or Cu/S ratio of 1.8:1 (Figure S5), which should produce copper exchanged NCs that are stable under air-equilibrated media.\(^{31a}\) Even after a quasi-quantitative exchange, the samples remained stable
in solution, with no sign of aggregation, as evidenced by TEM and DLS characterization (Figure S5). Further confirmation of the quantitative CE reaction came from the X-ray diffraction patterns of the samples before and after the exchange reaction. Indeed, when working at a Cu/Se or Cu/S ratio of 1.8, a quantitative transformation from ZnSe to Cu$_{2-x}$Se, from ZnS to Cu$_{2-x}$S and from CuFeS$_2$ to Cu$_{2-x}$S NCs occurred, as shown by the characteristic diffractometer patterns of the exchanged Cu:NCs (Figure S5).

Finally, further proofs of NC transformation are found in the optical spectral changes: Cu$_{2-x}$Se and Cu$_{2-x}$S NCs, obtained upon CE exchange, exhibit a NIR localized surface plasmon resonance band (LSPR) that was not present in the spectra of the initial ZnSe and ZnS NCs (Figure S6). On the contrary, for CuFeS$_2$ NCs, the dielectric resonance at 496 nm attributed to the intermediate band formed by the presence of Fe,$^{[32]}$ disappears when an almost quantitative exchange occurs (Figure S6).

These data, all together, suggest that under the experimental conditions set by us, the copper CE reactions on the NCs occurred quantitatively, irrespective of the materials composition and the initial percentage of Cu to be exchanged (13% or almost 100%).

To verify the chemical stability of the Cu-exchanged NCs, after the CE reactions (total exchange Cu/Se or Cu/S fixed at 1.8, corresponding to about 90% of the fully stoichiometric Cu$_2$S), the Cu$_{2-x}$Se and Cu$_{2-x}$S NCs were kept in pure water or in human serum at 37 °C for well-defined times. Elemental analysis revealed that, in water, a minor copper leakage from the NCs started to occur only after 24h (around 5%, Figure S7). Copper release, however, was more pronounced in the human serum, as indeed 68% and 85% of the total copper on the Cu$_{2-x}$Se and Cu$_{2-x}$S NCs
respectively was released after 24 h. (Figure S8). Conversely, the copper leakage was lower when applying the same stability tests to partially-exchanged NCs (Cu/Se or Cu/S 13 %, Figure 2e).

For the first set of CE reactions with the $^{64}$Cu ions, the so called “hot” copper, we have therefore considered a partial CE reaction with a $^{64}$Cu/Se or $^{64}$Cu/S ratio corresponding to 13%. For each radiolabeling experiment, we used 37 MBq of a $^{64}$CuCl$_2$ solution in 10 mM HCl (specific activity 50 GBq/µmol). Prior to the addition of the radiocopper solution, the pH of the $^{64}$Cu solution had been adjusted to pH 5-6 with a NaOH solution. Due to the low amount of total copper in this radiolabeled solution (7.4×10$^{-10}$ moles), the amount of NCs was reduced with respect the one used in the cold experiments, while keeping the same ratio of copper/sulfur (or copper/selenium) to 13 %. In a typical reaction, 20-10 µL of the NC solution (0.3-0.6 mM in sulfur or selenium, 0.9-0.5 µg of NCs) was diluted in 100 µL of an MES buffer (0.05 M at pH 5.5), then it was mixed with 2 µL of AA (0.1 M) and finally a $^{64}$Cu solution was added to the reaction mixture. In this case, the CE reaction temperature was set at 50 °C and the reaction was run for 1h. The radiolabeling yield was assessed via radio-TLC, using glass microfiber chromatography paper impregnated with silica gel (iTLC-SG) as stationary phase and 0.1 M EDTA as mobile phase (Figure 2b). The radio-TLC run revealed that, in the presence of NCs, the radioactivity was mostly located at the origin, while in case of free $^{64}$Cu the spot migrated with the solvent front. The integration of the TLC peaks at the deposition point and at the front of the solvent enabled us to estimate the radiochemical yield (RCY) that is associated with the NCs. For all types of NCs, quantitative radiolabeling was found (RCY > 99 % ~just after the reaction; Figure 2b and Figure S9).

The stability of the radiolabeled NCs was tested by incubating the reaction mixture (after CE reaction) in EDTA 0.1 M for 20 minutes (Figure S9). The integration of peaks from radio-TLC
chromatogram of radio-TLC showed that less than 2% of $^{64}$Cu was released, meaning that non-specific Cu absorption by the polymer at the surface of NCs could be excluded (the adsorbed $^{64}$Cu ions would be easily washed out by EDTA) and a high RCY can be achieved with this CE protocol (Figure 2c).

To verify the colloidal stability of $^{64}$Cu:NCs during the CE reaction and after the following cleaning steps (which could consequently cause a loss of the radiolabeled NCs), all plastic parts involved in the radiolabeling (cartridge, vials, pipette tips, etc.) were measured with a gamma counter (Figure S4 and S10a). Most of the activity was recorded in the NC fraction (53% to 57%), but some radioactivity (5.9 to 16%) was also found on the amicon centrifuge cartridge, indicating a minimal loss of radiolabeled NC. Another significant amount of activity (ca. 30%) was collected in the empty plastic vials and pipette tips, indicating a partial adhesion of the NCs to the plastic materials. Overall, the sum of the recovery activity was close to 97% of the initial one. We used a similar test not only for NCs with multidentate ligands (Figure 1) but also for NCs coated with a mono-dentate PEG-thiol polymer (SH-PEG2000-OCH$_3$) and exposed to the same CE reaction protocol. In the case of coating the NCs with SH-PEG2000-OCH$_3$, 60% of the radiolabeled material adhered to the amicon cartridge, and just 3% of the radioactivity was recovered with the NC solution (Figure S4). This higher aggregation and instability of mono-dentate PEG coated NCs is likely caused by the ligand depletion from NCs surface upon the cation exchange reaction, as shown in our previous paper.[25] Thus the multi-anchoring polymer with cysteamine was preferred to a monodentate polymer for all the following studies, due to the higher colloidal stability during CE reactions, especially when using $^{64}$Cu-labeling.

Purified $^{64}$Cu:NCs were also tested in human serum to evaluate $^{64}$Cu leakage. The NCs were diluted in pure serum (200 µL) and incubated for 1 h or 24 h at 37 °C. The amount of $^{64}$Cu
that was released was determined via radio-TLC in EDTA 0.1 M (Figure S11). $^{64}\text{Cu}:\text{CuFeS}_2$ is quite stable for 1 h in the presence of human serum. On the other hand, a significant $^{64}\text{Cu}$ release is observed for $^{64}\text{Cu}:\text{ZnS}$ and $^{64}\text{Cu}:\text{ZnSe}$ after just one hour, but especially after 24 h. It is worth noting that the $^{64}\text{Cu}$ leakage is comparable to the leakage that was measured by ICP when carrying out cold copper reactions (Figure 2d and 2e). Data from both tests suggest a lower copper release for $^{64}\text{Cu}:\text{CuFeS}_2$ NCs in comparison to $^{64}\text{Cu}:\text{ZnSe}$ and $^{64}\text{Cu}:\text{ZnS}$ NCs (Figure 2e).

These CE reactions were first performed at 50°C. However, such temperature can be harmful for radiolabeling of NCs bearing temperature-sensitive biomolecules (e.g., antibodies, peptides, etc.). We have therefore repeated the CE reaction at body temperature 37 °C and used experimental conditions similar to those of the first set of reactions performed at 50°C (Figure 1). As expected, at 37°C, for all NC compositions, lower RCYs were found as compared to the reaction performed at 50°C (RCYs between 30 % and 50 % with respect to almost 100 %) (Figure 3 and Figure S11).

In this case, however, the lower RCY was not related to the loss of stability of the NCs, as indeed only negligible fractions of radioactivity were found either in the reaction vials or in the amicon cartridge after purification, but it is clearly related to the lower temperature used for the CE reaction. However, even at 37°C, by increasing the amount of NCs, the RCY improved, and the NCs aggregated less on the amicon cartridge and vial (Figure 3). Keeping constant the overall feeding $^{64}\text{Cu}$ activity, by varying the amount of NCs from 0.2 to 6-9 μg, we could tune the specific activity of NCs from 60-100 TBq/g to 2-3 TBq/g. A specific activity of 60-100 TBq/g NC is a record value, never reported so far (Table S2). However, the radiolabeling stability of the NCs in the human serum was strongly compromised and a substantial leakage of $^{64}\text{Cu}$ was measured at
24h (Figure 3). On the other hand, for local tumor treatment using intra-tumoral injection, NCs at higher specific activities (60-100TBq/g) may be used, since the $^{64}$Cu:NCs are trapped in the tumor.

Instead, when investigating the $^{64}$Cu:NCs with a specific activity as low as 2-3 TBq/g, there was an increase of stability in human serum for all the three material compositions (Figure 3). The $^{64}$Cu percentage that was released into the serum media ranged from 11% to 24% after 24 h incubation. These leakage values are in line with those of other radiolabeling approaches. It is worth to highlight that, usually, the specific activity of radiolabeled NCs is not considered. But this value can be easily calculated based on the total activity associated to the NC fraction and knowing the corresponding amount of the nanomaterial used in the radiolabeling experiment (Table S2, ESI). We would like to stress that, when using our CE protocols, even for activities as low as 2-3 TBq/g, the specific activity is still higher than that of other $^{64}$Cu-labeled NCs prepared using a surface-bound chelating ligands$^{[33]}$ and some chelator-free radiolabeled NCs (Table S2, ESI)$^{[15a, 24c]}$

As previously mentioned, for ZnSe and ZnS NCs complete cation exchange on those materials leads to the formation of copper deficient chalcogenides, with the appearance of a LSPR band in the near infrared. Even in case of partial exchange, upon 13% of Cu exchange, the absorption spectra are modified (Figure 4): The initial ZnSe and ZnS NCs, that are not photothermally active, upon slightly exchange with Cu showed a thermal response upon 808 nm laser irradiation. Instead, chalcopyrite CuFeS$_2$ NCs that are already photo-thermally active prior to the Cu exchange$^{[26b]}$ upon replacement of 13% of Cu showed a slight shift in the absorption spectrum, but the partially exchanged Cu:CuFeS$_2$ NCs are still thermally active under laser irradiation (Figure 4c). These spectral features also guarantee that the Cu exchanged NCs are all thermally active
under a IR laser irradiation, as recorded by the temperature changes under an IR camera (Figure 4). The temperature that could be reached, after the Cu exchange by the same NC dose, was higher for Cu:CuFeS$_2$ NCs than for Cu:ZnS and Cu:ZnSe NCs (76°C for Cu:CuFeS$_2$ vs. 33°C for Cu:ZnSe and 26 °C for Cu:ZnS). For this reason, we have selected the CuFeS$_2$ NCs, and on this sample the exchange was performed with $^{64}$Cu ions (ratio $^{64}$Cu/NCs at 0.2%, see ESI for more details).

Under a NIR-laser irradiation (808 nm, 2 W/cm$^2$), on a concentrated spot of the $^{64}$Cu/NCs sample, loaded on agarose gel phantom, a light purple spot appeared under the IR camera, indicating the temperature raise from room temperature (20°C) to 27°C (Figure 4d). At the same time, the radioluminography image shows that radioactivity was localized in the deposition point (Figure 4e). Given the temperature reached under laser irradiation even when only a tiny fraction of $^{64}$Cu ions (0.2%) is exchanged, these data highlight the possible use of the exchanged $^{64}$Cu:CuFeS$_2$ chalcopyrite NCs as radio- and photo-thermal therapeutic tools, in which the two therapeutic modalities can be combined by using a single nanoplatform.

To provide a proof of concept for such dual therapeutic effects, an *in vitro* cellular study was performed on two cancer cell lines, the human glioblastoma cell line (U87) and the human epidermoid carcinoma (A431). For these tests, the cell pellets (2 million cells) mimicking a tiny tumor mass were incubated with radiolabeled $^{64}$Cu:CuFeS$_2$ NCs or with $^{64}$CuCl$_2$ solution with or without the exposure to an IR laser irradiation of clinical use (808 nm and 1.7 W/cm$^2$ for 13 min) for 3 short cycles (Figure 5a).

During the laser exposure, the temperature of the pellet treated with $^{64}$Cu:CuFeS$_2$ NCs increased remarkably when exposed to the 808 nm laser. In contrast, the cell samples exposed to
The \( ^{64}\text{CuCl}_2 \) solution showed only a few degrees (3 °C) temperature variation due to laser light effect (Figure 5b). Interestingly, at 2 hours post-irradiation at 37 °C, the \( ^{64}\text{Cu} \) cellular uptake measured in terms of radioactivity associated to the cell pellet for the \( ^{64}\text{Cu}:\text{CuFeS}_2 \) NCs in the presence of the laser increased up to 70% with respect to the total \( ^{64}\text{Cu} \) dose initially administered (Figure 5c). This activity uptake percentage was certainly higher than that of the pellet sample treated with \( ^{64}\text{Cu}:\text{CuFeS}_2 \) NCs which was not exposed to IR irradiation (in this case only 45% of the radioactive dose was associated to the cell pellet when no laser was used, Figure 5c). Moreover, in this latter case, the radioactive percentage of uptake was very similar to that of the \( ^{64}\text{CuCl}_2 \) solutions, with or without laser exposure (ca. 45-47%, Figure 5c data in the Table). Reasonably, the hyperthermia effect induced by the photo-thermal treatment led to enhanced cell membrane permeability with consecutively enhanced uptake of NCs and, in turn, \( ^{64}\text{Cu} \) radioisotopes uptake associated to the NCs.

To evaluate the toxicity of the radioactive NCs, a viability test (presto blue) was carried out. For these trials, the same amount of radioactivity (37 MBq) was administered to the cells either in the form of \( ^{64}\text{CuCl}_2 \) solutions or as \( ^{64}\text{Cu}:\text{CuFeS}_2 \) NCs. After irradiation and 2 hours incubation at 37°C (Figure 5a), the cells were re-cultured and the viability was measured at well-defined time points post-incubation and compared to cells exposed to radioactive NCs without NIR irradiation. After 16 h, while \( ^{64}\text{CuCl}_2 \) alone could kill 40% (U87) and 45% (A431) of the cells, the addition of laser irradiation to the \( ^{64}\text{CuCl}_2 \) solutions did not cause any additional cytotoxic effects to the cell populations (viability was of 40% for U87 and 47% for A431 and very similar to the \( ^{64}\text{CuCl}_2 \) solutions with no laser exposure). Radiolabeled \( ^{64}\text{Cu}:\text{CuFeS}_2 \) NCs alone could eliminate 45% (U87) and 51% (A431) of the cells, a percentage slightly higher than that of free \( ^{64}\text{CuCl}_2 \) solutions. In case of additional laser irradiation, the combination of radiotoxicity and
photo-thermal heat effects mediated by $^{64}$Cu:CuFeS$_2$ could significantly accelerate the cell killing (percentage of viability as low as less than 10% indicates remarkable toxicity effects). The difference in cytotoxicity appears already after 16 h of incubation (93% of cell elimination for U87 and 92% for A431) and it manages to reduce the cell densities to less than 10% viability up to 48 h (Figure 5d and 4e). These data indicate that damages by laser and radioactivity was so critical that the cells were not able to recover even after the half-life of the $^{64}$Cu radioisotopes was significantly reduced (the half life of $^{64}$Cu is 12.7 h and of the average dose associated to the cells the radioactivity at 48 h should be in the range of 1.1–1.85 MBq).

We also wondered about the intrinsic toxicity of the NCs at a dose range close to the amount of NCs needed for the radiolabeling experiments on the cells. To evaluate this toxicity, pristine ZnS, ZnSe and CuFeS$_2$ NCs were administered to the two tumor cells (U87 and A431 cells also used for the dual modal therapeutic experiments and the presto blue proliferation assays was assessed (Figure S12). The NCs were added to the cell culture media, and incubated at 37 °C for 24h. On both cell lines, for all the three material’s compositions, toxicity was not significant as the viability was more than 80% (Figure S12). These data suggest that the doses employed for the radio experiments on cells using the three different compositions of NCs are safe.

3. Conclusions

We have here set simple post-synthesis cation exchange (CE) protocols to prepare $^{64}$Cu-radiolabeled NCs, on Cd-free chalcogenide NCs made of ZnSe, ZnS and CuFeS$_2$ NCs. This method relies on the exchange of the pristine cations of the NCs with $^{64}$Cu ions when employing physiologically stable chalcogenide NCs. The CE reactions were performed by simply mixing the NCs in MES buffer (pH 5.5) in the presence of ascorbic acid (used as a mild reducing agent for
$^{64}\text{CuCl}_2$). Radiolabeling can occur at 50 °C or at 37 °C and in a short time (60 minutes). The radiolabeled NCs had a high radiochemical yield (RCY, >99%) and high radiochemical purity (close to 100%). The high RCY enables to use these NCs without any post-radiolabeling purifications steps. This is advantageous, as it reduces the time lag between $^{64}\text{Cu}$ production and the use of the $^{64}\text{Cu}$-NCs, particular suitable for $^{64}\text{Cu}$ having a half-life of about 13 hours. Moreover, by fixing the amount of $^{64}\text{Cu}$ and by tuning the NCs dose we could tune the specific activity from 103 TBq/g to 2 TBq/g NC, reaching the highest and unprecedented specific activity when using a very tiny dose of NC material (µg amounts). Finally, being the radiolabeling protocol the last step of the preparation pipeline and being performed on physiologically stable NCs, this post-synthesis radio-protocol could be easily implemented in hospitals.

Among the three NC compositions studied, $^{64}\text{Cu}:\text{CuFeS}_2$ chalcopyrite NCs maintained their photo-thermal properties even after the partial radiolabeling reaction (13% exchange) while producing remarkable ionizing effects. This may pave the way for the production of a double-therapeutic tools in which the intrinsic photo-thermal properties of the material and the radiation from the radionuclide are combined in a single nano-object as here shown in vitro on two cancer cell lines. At the same time, negligible intrinsic material toxicity has been recorded at the µg dose of NCs used for the dual therapeutic effects.

With regard to the stability of $^{64}\text{Cu}$:NCs in serum, it is worth to highlight that tuning the $^{64}\text{Cu}/\text{NC}$ ratio could be a useful parameter to be adjusted based on the aimed application of the NCs. At lower specific activity it is possible to have serum-stable radiolabeled $^{64}\text{Cu}$:NCs useful for imaging applications (PET). Instead, at higher specific activity serum stability is compromised. However these fully exchanged $^{64}\text{Cu}$:NCs may be more suitable for local tumor treatment upon intratumoral injection as in this treatment no direct exposure to serum is expected while reaching
a high specific activity and photo-thermal hyperthermia with a very tiny µg dose of NCs is achievable.

Supporting Information

The nanocrystals synthesis, the water transfer protocols, the cation exchange reactions conditions for non-radiolabeled Cu ions and for ⁶⁴Cu radiolabeled ions with the protocol at 37 and 50 °C, the nanocrystals characterization, the cellular study are reported step-by-step and in detail in Supporting Information available from the Wiley Online Library or from the author.

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**Figure 1**  

a) Cys-PIMA-PEG polymer ligand structure used for the water transfer of all NCs involved in this study.  
b) Schematic sketch of the CE reaction on the water-dispersible NCs.  
c), f), i) TEM images of water-dispersible NCs used for the CE reaction.  
d), g), l) DLS hydrodynamic sizes (in number weighted) before (black line) and after (red line) the partial (13%) Cu exchange reaction showed no change in size, indicating the absence of aggregation after CE.  
e), h), m) Elemental analysis to follow the partial CE reaction (13%): Cu, Se, S, Zn or Fe element quantification, after CE reactions, reported as a percentage of the initial elements on the NC fraction (Cu:ZnSe, Cu:ZnS and Cu:CuFeS$_2$) and on the washing solutions (filtrate) after NCs separation for e) ZnSe, h) ZnS and m) CuFeS$_2$ NCs.
Figure 2: a) Sketch of the $^{64}$Cu radiolabeling via CE reaction on the water-dispersible NCs b) Radio-TLC of the crude of 13% CE reactions (after 1h of incubation at 50 °C) for $^{64}$Cu:ZnS, $^{64}$Cu:ZnSe and $^{64}$Cu:CuFeS$_2$ (EDTA 0.1M as a mobile phase); free $^{64}$CuCl$_2$ is also spotted as a reference control. c) Integration of peaks from radio-TLC chromatogram of NCs previously exposed for 20 min to an EDTA 0.1 M solution; most of the Cu is still associated to the NCs fractions, indicating stable radiolabeled NCs products. d) Results from radio-TLC integration (EDTA 0.1M mobile phase) of $^{64}$Cu released upon incubation of $^{64}$Cu:ZnS, $^{64}$Cu:ZnSe and $^{64}$Cu:CuFeS$_2$ in human serum at 37 °C for 1 h and 24 h. e) Copper released upon 24 h serum incubation of Cu:NCs; amicon wash was performed using EDTA 0.1 M solution. The free copper incubation experiment is also reported.
Figure 3. Scheme of amicon purification upon CE reaction (a). Set of CE reactions performed at 37°C on different NCs compositions. To tune the specific activity, by keeping the $^{64}$Cu activity (18.5 MBq) the amount of NCs was varied in the range from 0.2 to 9.4 µg. Radio-distribution test: Monitoring the radioactivity on the reaction components after CE reactions as a percentage of the initial $^{64}$Cu activity: NCs (green); washing solutions (blue); amicon filters (orange); and the vials (grey). Stability of $^{64}$Cu:NCs in serum. $^{64}$Cu leakage is given as percentage of the total radioactivity in a single TLC strip. Radio-stability test: Radio-TLC integration after incubation for 1h or 24h for $^{64}$Cu:ZnS (b), $^{64}$Cu:ZnSe (c) and $^{64}$Cu:CuFeS$_2$ (d) NCs fraction after CE reaction.
**Figure 4 a)** Absorption spectra of ZnSe and Cu:ZnSe NCs, **b)** ZnS and Cu:ZnS NCs and **c)** CuFeS\(_2\) and Cu:CuFeS\(_2\), before (black line) and after (red line) undergoing a 13% Cu exchange; the inset panel is a IR image of the Cu:NCs after 3 min of continuous exposure to an 808 nm laser at 2 W/cm\(^2\). A digital picture (panel **d**) and the radioluminography (panel **e**) of a tiny drop (35 μL) of \(^{64}\)Cu:CuFeS\(_2\) NCs (exchanged at 0.2%) loaded on agarose gel (0.5% in PBS 1X). Also, the IR image taken after 20 min of exposure to an 800 nm laser at 2 W/cm\(^2\) is shown (**f**).

**Figure 5 a)** Schematic figure depicting the 808 nm laser irradiation on cell pellet treated with either \(^{64}\)CuCl\(_2\) or \(^{64}\)Cu:CuFeS\(_2\) NC solution. **b)** Temperature profiles upon laser irradiation for a cell pellet treated with a \(^{64}\)CuCl\(_2\) (top) or with a \(^{64}\)Cu:CuFeS\(_2\) NC solution (bottom). **c)** Radio-labeling test for cell uptake on two different cell lines for \(^{64}\)CuCl\(_2\) or \(^{64}\)Cu:CuFeS\(_2\) NC with and
without the effect of laser irradiation. Cell viability upon the dual action of radiotherapy and laser treatment on d) U87 cells and e) A431 cells.
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Here we report the first examples of cation exchange reactions used for quantitative radiolabelling with $^{64}$Cu aqueous stable metal chalcogenides of ZnSe, ZnS and CuFeS$_2$ nanocrystals setting protocols readily transferable to the clinic. We also demonstrate the exploitation of radiolabelled-nanocrystals for dual therapy combining internal radiotherapy and photo-therapy.

**Keywords:** cation exchange reactions, photo-thermal therapy, internal radiotherapy, dual therapy, metal chalcogenides nanocrystals

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**Title:** Cation exchange protocols to radiolabel aqueous stabilized ZnS, ZnSe and CuFeS$_2$ nanocrystals with $^{64}$Cu for dual radio- and photo-thermal therapy
Supporting Information

Cation exchange protocols to radiolabel aqueous stabilized ZnS, ZnSe and CuFeS<sub>2</sub> nanocrystals with $^{64}$Cu for dual radio- and photothermal 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$_2$O$_2$/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).

\[
Specific\ activity\ (TBq/g) = \frac{Activity\ (TBq)}{mass\ of\ NCs\ (g)} \times RCY
\]

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).
ZnSe NCs were synthesized accordingly to a previous published work.\textsuperscript{[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 1 h. 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\textsubscript{2} NCs were synthetized following a protocol that has already been reported by our group.\textsuperscript{[2]} ZnS NCs were synthesized following the hot-injection protocol reported by Joo \textit{et al.}\textsuperscript{[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\textsubscript{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\textsubscript{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.

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

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

|                  | 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) | $5.89 \times 10^{-19}$ | $2.14 \times 10^{-18}$ | $9.82 \times 10^{-19}$ |
| 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.

\[
\text{Conc of NCs (mol/L)} = \frac{\text{total mass of elements (} g \text{)}}{\text{MW of single NC (} g/mol \text{)}} \times \text{Volume (} L \text{)} \quad \text{Eq. S4}
\]
**S5 Cys-PIMA-PEG synthesis**

Cys-PIMA-PEG were synthesized based on a procedure that has already been reported in literature, 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.

**Figure S3** a) $^1$H-NMR spectra of Cys-PIMA-PEG in D$_2$O. 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-OCH₃ ligand exchange**

ZnSe was transferred into water using cys-PIMA-PEG in the presence of Zn(NO₃)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/nm²) was mixed with 60 mg of Zn(NO₃)₂•6H₂O. 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 CuFeS₂ were transferred in water following a similar protocol. In the case of CuFeS₂ NCs, no Zn(NO₃)₂ was added.

The ZnSe ligand exchange with SH-PEG-OCH₃ 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-OCH₃ (2000 g/mol, Rapp Polymere, containing 0.09 mmol of –SH group, corresponding to about 73 SH/nm²) was dissolved in 3 mL of ethanol and mixed with 100 mg of Zn(NO₃)₂•6H₂O. 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-OCH₃ 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-OCH₃ 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.
| Chelator Free | NPs (g) | Activity (GBq) | RCY (unit) | Specific activity (TBq/g) |
|--------------|---------|---------------|------------|-------------------------|
| 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 |
| A chelator-free multifunctional \(^{64}\)Cu-CuS nanoparticle platform for simultaneous micro-PET/CT imaging and photothermal ablation therapy\(^11\) | 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\(^{-3}\) | 1 | Value in the paper \((200\mu\text{Ci/mg})\) |
| 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\(_2\)Se\(_3\) 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 different surface coatings using embedded copper-$^{64}$\textsuperscript{[19]}.

| Our protocol       | Activity | Purity | Yield | Specific Activity |
|--------------------|----------|--------|-------|------------------|
| ZnSe-cys-PIMA-PEG  | $8.66\times10^{-7}$ | 0.037  | 1     | 43               |
| CuFeS\textsubscript{2}-cys-PIMA-PEG | $5.0\times10^{-7}$ | 0.037  | 1     | 74               |
| ZnS-cys-PIMA-PEG   | $5.85\times10^{-7}$ | 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.
Complete cation exchange reactions were carried out in a 96-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 CuCl₂ 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/cm²) for 3 min. The temperature was monitored using a thermocamera (Fluke Ti200).

|             | Vol NCs (µL) | Conc Se or S (M) | Vol CuCl₂ (µL) | Vol AA (µL) | Vol water (µL) |
|-------------|--------------|------------------|----------------|-------------|----------------|
| ZnSe        | 15           | 0.049            | 61             | 30          | 94             |
| ZnS         | 15           | 0.089            | 111            | 50          | 24             |
| CuFeS₂      | 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” CuFeS₂). Photothermal picture of NCs before and after Cu exchange upon 3 min irradiation with an 808 nm laser at 2 W/cm² (“b” ZnSe, “d” ZnS and “f” CuFeS₂).
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.

![Graph](image)

**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 blood, 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 CuCl₂, 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 $7.4 \times 10^{-10}$.

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

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

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

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 $4.05 \times 10^{-12}$. 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”

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

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.
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)).

|                | ZnSe MW 354700 g/mol | ZnS MW 1288700 g/mol | CuFeS$_2$ MW 591400 g/mol |
|----------------|----------------------|----------------------|---------------------------|
| Activity (MBq) | 37                   | 37                   | 37                        |
| Total Cu added (mol) | $7.4 \times 10^{-10}$ | $7.4 \times 10^{-10}$ | $7.4 \times 10^{-10}$ |
| Ratio Cu/S or Cu/Se | 13 %             | 13 %             | 13 %                        |
| Conc element (mM) |                     |                     |                           |
| Se 0.6          | Zn 0.6             | Cu 0.15            |
| Zn 0.6          | S 0.6              | Fe 0.15            |
| S 0.6           |                     | S 0.3              |
| Vol NCs (µL)   | 10                  | 10                  | 18                        |
| Mass NCs (g)   | $8.66 \times 10^{-7}$ | $5.85 \times 10^{-7}$ | $5.00 \times 10^{-7}$ |
| moles NCs      | $2.44 \times 10^{-12}$ | $4.54 \times 10^{-13}$ | $8.45 \times 10^{-13}$ |
| Conc NCs (mol/L) | $2.44 \times 10^{-7}$ | $4.54 \times 10^{-8}$ | $4.70 \times 10^{-8}$ |
| Specific activity (TBq/g) | 43              | 63              | 74                        |

Table S2 Characteristics and quantity of all NCs employed in each radiolabeling reaction.
Figure S9 Results from radio-iTLC integration of a) $^{64}\text{Cu}:\text{ZnS}$, b) $^{64}\text{Cu}:\text{ZnSe}$, c) $^{64}\text{Cu}:\text{CuFeS}_2$, radiolabeling at 50 °C for 1 h (RCY > 99%); d) $^{64}\text{Cu}:\text{ZnS}$ (1.5% $^{64}\text{Cu}$ release), e) $^{64}\text{Cu}:\text{ZnSe}$ (1% $^{64}\text{Cu}$ release), f) $^{64}\text{Cu}:\text{CuFeS}_2$ (< 1% $^{64}\text{Cu}$ release), challenging $^{64}\text{Cu}$-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$_2$. 
Radiolabeling reaction at different amount of nanocrystals

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 $1.32 \times 10^{-10}$. The concentration of NCs was calculated based on Eq. S4.

| Conc S/Se stock (mM) | Reaction 1 | Reaction 2 | Reaction 3 | Reaction 4 | Reaction 5 |
|----------------------|------------|------------|------------|------------|------------|
| Conc ZnSe NC (mol/L) | 8.1 $\times$ 10$^{-8}$ | 1.6 $\times$ 10$^{-7}$ | 3.3 $\times$ 10$^{-7}$ | 1.3 $\times$ 10$^{-6}$ | 2.6 $\times$ 10$^{-6}$ |
| Conc ZnS NC (mol/L)  | 1.5 $\times$ 10$^{-8}$ | 3.0 $\times$ 10$^{-8}$ | 6.0 $\times$ 10$^{-8}$ | 2.5 $\times$ 10$^{-7}$ | 4.9 $\times$ 10$^{-7}$ |
| Conc CuFeS$_2$ NC (mol/L) | 3.1 $\times$ 10$^{-8}$ | 6.2 $\times$ 10$^{-8}$ | 1.2 $\times$ 10$^{-7}$ | 5.1 $\times$ 10$^{-7}$ | 1.0 $\times$ 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)

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

| 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 $\times$ 10$^{-8}$ | 10           | 6.6 %           | 8.1 $\times$ 10$^{-13}$ | 2.9 $\times$ 10$^{-7}$ | 18.5          | 63                       |
| Reaction 2        | 1.6 $\times$ 10$^{-7}$ | 10           | 3.3 %           | 1.6 $\times$ 10$^{-12}$ | 5.8 $\times$ 10$^{-7}$ | 18.5          | 32                       |
| Reaction 3        | 3.3 $\times$ 10$^{-7}$ | 10           | 1.7 %           | 3.3 $\times$ 10$^{-12}$ | 1.2 $\times$ 10$^{-6}$ | 18.5          | 15                       |
| Reaction 4        | 1.3 $\times$ 10$^{-6}$ | 10           | 0.4 %           | 1.3 $\times$ 10$^{-11}$ | 4.8 $\times$ 10$^{-6}$ | 18.5          | 3.9                      |
| Reaction 5        | 2.6 $\times$ 10$^{-6}$ | 10           | 0.2 %           | 2.6 $\times$ 10$^{-11}$ | 9.4 $\times$ 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.
Table S5 Volume, concentration, moles and mass of ZnS, Cu/S ratio and specific activity of the radiolabeling reactions upon variation of NCs amount

| Reaction | Conc NCs (mol/L) | Vol NCs (µL) | Ratio Cu/S (%) | mol NCs | mass NC (g) | Activity (MBq) | Specific activity (TBq/g) |
|----------|------------------|--------------|----------------|---------|------------|---------------|--------------------------|
| Reaction 1 | $1.5 \times 10^{-8}$ | 10 | 6.6 % | $1.5 \times 10^{-11}$ | $1.9 \times 10^{-7}$ | 18.5 | 97 |
| Reaction 2 | $3.0 \times 10^{-8}$ | 10 | 3.3 % | $3.0 \times 10^{-11}$ | $3.9 \times 10^{-7}$ | 18.5 | 47 |
| Reaction 3 | $6.0 \times 10^{-8}$ | 10 | 1.7 % | $6.0 \times 10^{-11}$ | $7.8 \times 10^{-7}$ | 18.5 | 23 |
| Reaction 4 | $2.5 \times 10^{-7}$ | 10 | 0.4 % | $2.5 \times 10^{-12}$ | $3.2 \times 10^{-6}$ | 18.5 | 5.8 |
| Reaction 5 | $4.9 \times 10^{-7}$ | 10 | 0.2 % | $4.9 \times 10^{-12}$ | $6.3 \times 10^{-6}$ | 18.5 | 2.9 |

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.
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 S4a). 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% CO₂. 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% CO₂. For the non-laser controls, a solution of $^{64}$CuCl₂, 37 MBq (110 µL) (with a pH previously adjusted at pH 6) was added to the cell pellet, while for $^{64}$Cu:CuFeS₂, 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/cm² 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 CuFeS₂, 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 CuFeS₂, ZnS and ZnSe NCs respectively while the green arrows indicate the concentration of the NCs used for radiolabeling.
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