Therapeutic Pretargeting with Gold Nanoparticles as Drug Candidates for Boron Neutron Capture Therapy

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Boron neutron capture therapy (BNCT) is a binary approach for cancer treatment in which boron-10 atoms and thermal neutrons need to colocalize to become effective. Recent research in the development of BNCT drug candidates focuses increasingly on nanomaterials, with the advantages of high boron loadings and passive targeting due to the enhanced permeability and retention (EPR) effect. The use of small boron-rich gold nanoparticles (AuNPs) in combination with a pretargeting approach is proposed. Small sized polyethylene glycol–stabilized AuNPs (core size 4.1 ± 1.5 nm), are synthesized and functionalized with thiolated cobalt bis(dicarbollide) and tetrazine. To enable in vivo tracking of the AuNPs by positron emission tomography (PET), the core is doped with [64Cu]CuCl2. For the pretargeting approach, the monoclonal antibody Trastuzumab is functionalized with trans-cyclooctene-N-hydroxysuccinimide ester. After proving in vitro occurrence of the antibody conjugation onto the AuNPs by click reaction and the low toxicity of the AuNPs, the boron delivery system is evaluated in vivo using breast cancer xenograft bearing mice and PET imaging. Tumor uptake due to the EPR effect can be witnessed with ≈5% injected dose (ID) cm⁻³ at 24 h postinjection, but with slower clearance than expected. Therefore, no increased retention can be observed using the pretargeting strategy.

1. Introduction

Cancer remains one of the leading causes of death worldwide.[1] In spite of recent advances, the development of therapeutic strategies capable to selectively actuate in the tumor while sparing healthy organs and tissues is urgently required. Boron neutron capture therapy (BNCT),[2] a binary approach to cancer therapy in which two nontoxic components need to colocalize to become effective, fulfills this criterion. In BNCT, Boron-10 (¹⁰B) atoms selectively delivered to tumor tissue are irradiated with neutrons. The interaction of the incident neutrons with the stable ¹⁰B atoms triggers a nuclear reaction which results in the formation of recoil lithium ions and α-particles. These have high linear energy transfer (LET) and a range within the diameter of a single cell (5–9 µm),[3] generating thus localized cellular damage which results in tumor-cell death.

Although BNCT was first proposed more than 80 years ago,[2] only two boronated compounds are currently used in clinical trials: (L)-4-dihydroxy-borylphenylalanine, commonly known as boronophenylalanine (BPA); and sodium mercaptoundecahydro-closo-dodecaborate, commonly known as sodium borocaptate (BSH).[4] These, however, show low tumor selectivity and are efficacious only in some cancer types. The lack of success in the development of...
promising drug candidates, together with the inconvenience of conducting clinical trials close to nuclear reactors, temporarily discouraged the development of BNCT. However, two major advances have opened new avenues for this promising therapeutic modality: first, the development of biomedical cyclotrons capable to generate high intensity neutron beams,[5] which enable treating patients in a friendly environment and at lower cost; second, the emergence of nanotechnology, which has provided the possibility of boron-rich nanomedicines capable to passively accumulate in tumor tissue by taking advantage of the enhance permeability and retention (EPR) effect. As a result, nanosized materials including liposomes,[6] iron oxide nanoparticles,[7] carbon[8] and boron nitride[9] nanotubes, and gold nanoparticles,[10] among others, have been proposed, synthesized, and eventually evaluated as boron carriers.

The size of the nanomaterials, together with surface charge and functionalization, plays a pivotal role in their biodistribution after intravenous administration, and severely affects the degree of accumulation in the tumor. In general terms, large particles show slow distribution with accumulation in organs with high presence of the mononuclear phagocytic system (MPS), such as the liver, the spleen, and the lungs, which may cause local toxicity and undesired side effects. Contrarily, ultrasmall particles below the glomerular filtration threshold of the kidneys (∼8 nm) are likely to be rapidly eliminated via urine.[11] Hence, they are less prone to show localized off-target toxicity, although bioavailability and hence tumor accumulation are severely compromised.

In the pursuit of a therapeutic strategy capable of achieving high tumor accumulation and fast clearance, we recently introduced a pretargeting strategy (Figure 1a),[12] previously applied in the fields of nuclear imaging and radiation therapy.[13] In our approach, a tumor targeting, trans-cyclooctene (TCO) functionalized monoclonal antibody (mAb) was administered first. After accumulation in tumor tissue and clearing from blood (24 h), tetrazine (Tz)-functionalized boron-rich carbon-based nanoparticles (particle diameter = 3–10 nm) were injected. The on-site click reaction between the TCO-mAb and the Tz-functionalized nanoparticles led to enhanced tumor retention when compared to the nanoparticles injected alone, without preinjection of the antibody. In spite of the positive results, maximum nanoparticle uptake in the tumor was ≈3% of injected dose per gram (%ID g⁻¹), mainly due to the extremely fast clearance of the nanoparticles. These results suggested the need for developing other boron-rich nanoplatforms with slower clearance. Among all alternatives, gold nanoparticles are particularly interesting, as gold is chemically inert and nontoxic, both shape and size can be easily tuned and surface properties and functionalization can be easily modulated due to the well-defined surface chemistry of gold.[14]

Herein, we report the synthesis, characterization, and in vivo evaluation of boron-rich gold nanoparticles using a pretargeting strategy. Based on our previous works,[10b] we synthesized small-sized gold nanoparticles (AuNPs; core size = 4.1 ± 1.5 nm) stabilized with polyethylene glycol and bearing cobalt bis(dicarbollide) (COSAN) units and Tz moieties on the

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**Figure 1.** a) Schematic representation of the pretargeting strategy using small boron-rich nanoparticles (NPs). The TCO-functionalized antibody (TCO-mAb) accumulates in the tumor 1). After clearance 2), boron-rich, tetrazine functionalized NPs 3) are injected. Those reaching the tumor undergo bioorthogonal click reaction and are selectively retained, while nonreacted particles are cleared 4). At the final stage, neutron irradiation can be applied; b) schematic representation of a small, spherical gold nanoparticle stabilized with polyethylene glycol (PEG) and functionalized with tetrazine (Tz) and thiolated cobalt bis(dicarbollide) (COSAN-SH); chemical structure of COSAN-SH is shown on the right.
surface (Figure 1b). Successful radiolabeling could be achieved by incorporation of the positron emitter copper-64 (\(^{64}\text{Cu}\)) within the gold core. The capacity of the newly developed nanosystem to selectively accumulate in the tumor via EPR effect, combined with a pretargeting strategy, was finally evaluated in a xenograft mouse model of breast cancer using positron emission tomography (PET) imaging.

2. Results and Discussion
2.1. Functionalization of the mAb Trastuzumab with TCO

Our studies were carried out in a breast cancer xenograft mouse model, generated by subcutaneous injection of human epidermal growth factor receptor (HER2) positive BT-474 cells in immunodeficient mice (see below). Hence, Trastuzumab, an antibody that selectively binds to HER2 and is clinically used to treat HER2\(^+\) breast and stomach cancer,\(^{[15]}\) was selected.

The selected antibody, Trastuzumab, was functionalized with a TCO ligand to enable the in vivo click reaction to the tetrazine-functionalized AuNPs. With that aim, the commercially available TCO-NHS was used and randomly conjugated to freely available amines in the antibody (Figure 2a). The conditions used (50–55 eq. excess of TCO-NHS with respect to the antibody, 60 min incubation at room temperature, \(8.6 < \text{pH} < 9.1\), mAb concentration of 3 mg mL\(^{-1}\)) led to an average conjugate of about two TCO per mAb, as determined by photometric titration (see the Experimental Section for details).

Figure 2. a) Illustration of the functionalization of the mAb with TCO-NHS by reaction with the free amino groups of lysine residues; b) Lindmo cell binding plot of \(^{[89}\text{Zr}]\)Trastuzumab on BT-474 cell; c–f) FACS studies, to monitor cell binding capability of TCO conjugated Trastuzumab; c,d) gating strategy applied in the flow cytometry study: live cells were electronically gated based on the forward and side scatter parameter c) and the not-single events leaved out based on forward area and height scatter parameters d); e,f) histogram of fluorescent BT-474 population with control (nolabeled): e) different concentrations of Trastuzumab (30 min incubation); f) different incubation times of Trastuzumab (0.1 mg mL\(^{-1}\)).
In order to ensure preserved binding capacity of the functionalized mAb, Lindmo assay, and flow cytometry studies based on fluorescence-activated cell sorting (FACS) were carried out. The determined immunoreactive fraction by Lindmo assay was close to 90% (Figure 2b), and flow cytometry experiments showed, with a concentration of 0.1 mg mL$^{-1}$ and an incubation time of only 5 min, that over 80% of cells carried Trastuzumab (Figure 2c–f). Hence, both experiments revealed good mAb-receptor binding capacity after conjugation with TCO.

2.2. Synthesis, Characterization, and Biocompatibility of Gold Nanoparticles

Previous studies carried out in our group with $^{124}$I-labeled COSAN-functionalized gold nanoparticles with a core size of $\approx$20 nm demonstrated high accumulation in organs with high presence of the MPS, such as the liver and the spleen, together with low accumulation in the tumor probably due to low bioavailability.[10b] These results suggested that, in order to favor a fast clearance and prevent long residence time in these organs, there was the need to decrease particle core size. With this in mind, we decided in this study to prepare AuNPs with smaller core size. Additionally, the labeling strategy was also modified, as $^{124}$I is an inconvenient radionuclide due to low availability. Based on previously reported works,[17] we decided to use a synthetic route enabling the incorporation of copper-64 (positron emitter, $t_{1/2} = 12.7$ h) into the gold core, as this strategy was anticipated to guarantee radiochemical integrity of the AuNPs after intravenous administration. The approach consisted of synthesizing copper-alloyed particles (Figure 3a), as translation to radiolabeling conditions is straightforward by simple spiking stable copper with copper-64. Experimentally, a solution of chloroauroic acid (HAuCl$_4$) and CuCl$_2$ in water was reduced with sodium borohydride (NaBH$_4$) and stabilized with thiolated amino-polyethylene glycol (HS-PEG$_{5k}$-NH$_2$). A color change could be observed during the addition of NaBH$_4$ from the light yellow of HAuCl$_4$ to a dark red, as typically observed for AuNPs. After purification and washings, tetrazine moieties were anchored to the AuNPs surface, in order to enable pretargeting. With that aim, the NHS-activated ester, which readily reacts with free amino groups available at the terminus of the

![Figure 3](image-url)

**Figure 3.** AuNPs synthesis and characterization: a) Schematic representation of synthesis and functionalization of AuNPs; b) UV–VIS spectra of the PEG-amine stabilized AuNPs core (red), functionalized with tetrazine (blue, max absorbance at 270 nm), and functionalized with tetrazine and COSAN (black, max absorbance at 315 nm); c) size distribution ranges as determined by DLS (intensity distribution); red: nonfunctionalized, PEG-amine stabilized AuNPs; black: tetrazine- and COSAN-functionalized AuNPs. d) Representative TEM images to visualize shape and size of AuNPs; e) histogram of core diameter distribution ($n = 670$ particles). In red, the Gaussian curve fitted to experimental data.
PEG chains, was used. Sufficient incorporation to yield subsequent click reaction was achieved (see below).

The ultimate goal of our strategy was to design AuNPs with application in BNCT. The cobalt bis(dicarbollide) anion \([3,3'-\text{Co}((\text{C}_2\text{B}_9\text{H}_{11})_2)]^+\), commonly known as COSAN, and consisting of a central cobalt (Co\(^{3+}\)) atom sandwiched between two \(\eta^5\)-bonding \([\text{C}_2\text{B}_9\text{H}_{11}]^-\) moieties, was selected. The main reasons behind this selection were the remarkable thermal, kinetic, and photochemical stabilities,\(^{[18]}\) the low toxicity both in vitro and in vivo,\(^{[19]}\) and its previous use in the development of radiotherapeutics and BNCT agents.\(^{[20]}\) In order to enable the attachment of COSAN on the surface of gold particles taking advantage of the thiol-gold chemistry, a thiolated derivative was synthesized as previously described.\(^{[10b]}\) Efficient incorporation of COSAN on the AuNPs was achieved by 1 h incubation at room temperature, as confirmed by combining different techniques (see below).

The multifunctionalized AuNPs were first characterized using UV–vis spectrophotometry, which is an easy and straightforward method to monitor each step of the synthesis and functionalization of the particles. If the core of the AuNPs is formed correctly, the typical plasmon, dependent on shape and size of AuNPs, is visible. For the particles in hand the surface plasmon resonance was observed at \(\approx 500\) nm (\(\lambda = 500\) nm for AuNP; \(\lambda = 501\) nm for Tz-functionalized AuNP; \(\lambda = 508\) nm for Tz- and COSAN-functionalized AuNPs), as expected for AuNPs with average core diameter below 5 nm (Figure 3b). The incorporation of the COSAN and tetrazines moieties was confirmed by the presence of additional peaks at \(\approx 315\) and 270 nm, respectively (Figure 3b).

Transmission electron microscopy (TEM) analysis was carried out to determine core size and shape (Figure 3d). Spherical particles with a core size of 4.1 ± 1.5 nm were obtained. To determine the hydrodynamic diameter of the particles, dynamic light scattering (DLS) analysis was performed (Figure 3c).

Intensity distribution curves showed a clear difference between the nonfunctionalized AuNPs and the COSAN- and tetrazine-functionalized particles, with an increase in the hydrodynamic diameter from 273 ± 4.0 to 396.6 ± 0.8 nm. The positive values obtained by zeta potential (\(\zeta\)-potential) measurements of PEG-stabilized NPs in ultrapure water (79 ± 9.4 mV) confirm the presence of free amino groups on the surface. After coupling of tetrazine, which results in a decrease of the free amino groups present on the surface, and COSAN, which bears a net negative charge, zeta potential values of \(-33.6 \pm 3.82\) mV were obtained.

Finally, and in order to get additional experimental proof of the presence of boron clusters on the AuNPs, the composition was investigated by inductively coupled plasma mass spectrometry (ICP-MS) analysis. With that aim, the concentrations of gold, boron, and cobalt were determined. Because each COSAN moiety contains one central cobalt atom sandwiched between two \([\text{C}_2\text{B}_9\text{H}_{11}]^-\) moieties, a boron to cobalt ratio of 18.1 was expected. ICP-MS analysis showed a ratio 18.2:1, thus confirming not only the presence of COSAN on the surface, but also its stability after attachment. Furthermore, the boron loading of the particles was determined to be \(195 \mu\text{g \cdot mg}^{-1}\) gold, showing high boron loadings of the particles and thus suggesting favorable chemical composition for eventual BNCT applications.

After the successful synthesis, functionalization, and characterization of the AuNPs, we experimentally proved the occurrence of the click reaction between the TCO functionalized Trastuzumab (TCO-mAb) and the tetrazine functionalized AuNPs. With that aim, agarose gel electrophoresis with a mixture of the two pretargeting components and respective controls was executed. The AuNPs derivatives were visible on the gel due to their red color. To visualize the mAb species after the electrophoresis, a staining of the gel with coomassie blue was performed. As control, each component was applied sole to the gel (Figure 4, lanes 1–2: Trastuzumab without and with TCO; lanes 6–7: AuNPs with and without tetrazine). Furthermore, a mix between either nonfunctionalized Trastuzumab and multifunctionalized AuNPs (Figure 4, lane 3) or functionalized Trastuzumab and AuNPs without tetrazine functionalization (Figure 4, lane 5) was applied. Good separation between the different species could be achieved. Additionally, the mixture of Trastuzumab-TCO and fully functionalized AuNPs (Figure 4, lane 4) showed a merged signal confirming the presence of both AuNPs and mAb, thus confirming that the click reaction took place.

Before moving to in vivo experiments, we evaluated the biocompatibility of the multifunctionalized AuNPs. With that aim, cytotoxicity studies on BT-474 breast cancer cells were performed. The cells were treated with 5, 10, 20, 60, 80, and \(120 \times 10^{-6}\) m (gold concentration) of AuNPs for up to 72 h. Analysis by standard 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay showed, with overall cell survivals over 90%, no induced cell death due to the particles in BT-474 cells, indicating negligible cytotoxicity (Figure S1, Supporting Information).

### 2.3. In Vivo Studies

We next moved to evaluate the suitability of the pretargeting strategy to accumulate boron atoms in the tumor. This strategy, using the same ligands but in a different NP type, has proven efficient to increase retention of the NPs in the tumor, confirming that the click reaction between the Trastuzumab-TCO and the tetrazine-functionalized NPs can occur in vivo.\(^{[12]}\) First, and in order to be able to track the AuNPs in vivo, a positron emitting radio nuclide copper-64 in the core.\(^{[17]}\) The synthesis of the radiolabeled particles was carried out following the procedure described as for the nonlabeled particles, but CuCl\(_2\) was spiked with radioactive \(^{64}\)Cu. To purify the AuNPs by spin filtration after the labeling, a \(1 \times 10^{-3}\) m EDTA (ethylenediaminetetraacetic acid) solution was used to remove all loosely bound \(^{64}\)Cu. Radio-thin layer chromatography (radio-TLC) studies after purification of the NPs showed the presence of only one peak at the seeding spot, confirming thus the radiochemical purity of the labeled NPs (Figure S2, Supporting Information). The radiochemical yield, calculated with respect to the starting amount of \(^{64}\)Cu, was close to 100%. Despite low, this value resulted in a sufficient amount of radioactivity to tackle subsequent in vivo studies. Furthermore, competitive stability tests showed that after 46 h at \(37^\circ\text{C}\) in a \(1 \times 10^{-3}\) m EDTA solution, 98% of \(^{64}\)Cu was still bound to the AuNPs, thus confirming that inclusion...
of the radionuclide into the crystal lattice of the gold core is an appropriate strategy (Figure S2, Supporting Information).

To evaluate the amount of NPs accumulating in the tumor, immunocompromised NOD/SCID mice were inoculated subcutaneously with human BT-474 breast cancer cells, and the animals were randomly distributed in three groups. For all experiments fully functionalized and $^{64}$Cu-radiolabeled AuNP were used ($\approx 190 \mu g$ gold with 1–2 MBq per mouse). In case of the pretargeting group (G1, $n = 4$) 100 $\mu g$ Trastuzumab-TCO per mouse was injected intravenously 24 h before the AuNPs. As control group (G2, $n = 3$) Trastuzumab without TCO conjugation was injected intravenously 24 h before the AuNPs. The last group, (G3, $n = 4$) also used as control, received only the administration of AuNPs.

PET-CT scans were performed $\approx 1, 6, 24$, and 48 h postinjection using the $\beta$- and X-cube microsystem of Molecubes. We first analyzed the third group to evaluate the biodistribution and tumor uptake of the AuNPs alone. Visual inspection of the images (Figure 5a) confirmed that the particles show a rather typical distribution for spherical gold nanoparticles. However, quantification data (Figure 5b) showed a significant uptake in the liver and the spleen. Yet, in organs such as the heart and the lungs, a steady decrease in the concentration of radioactivity can be observed over time, suggesting that the residence time of the NPs in blood is long. This slow distribution and the accumulation in the liver suggest that our ultrasmall-sized AuNPs (core size $\approx 4 \text{ nm}$) do not undergo renal clearance. This is, at first, surprising as it is known that small particles of below $\approx 8 \text{ nm}$ tend to follow renal clearance. However, the size of the particles increases due to the multifunctionalization; additionally, the hydrodynamic size is significantly larger ($\approx 40 \text{ nm}$ in diameter). This size is above the glomerular filtration, justifying the elimination route observed in our in vivo studies.

Maximum tumor uptake was achieved at $t = 24$ h postinjection, with values of $4.76 \pm 1.85 \% \text{ID cm}^{-3}$ (Figure 5c; and Table S1, Supporting Information), suggesting that there exists a passive accumulation of the NPs in the tumor, probably due to the EPR effect. A progressive, nonsignificant decrease in tumor uptake was observed afterward ($t = 48$ h), which suggests the retention of the NPs in the tumor tissue. The tumor accumulation was clearly visualized in PET images when represented in axial view (Figure 5d).

The analysis of the control (G2) and the pretargeting (G1) groups showed very similar biodistribution results at the whole body level (Figure 5b). Analyzing the tumor uptake, also very similar patterns were observed, with maximum uptake values of 4.42 $\pm$ 2.08 and 3.95 $\pm$ 0.38% ID cm$^{-3}$ for groups G1 and G2, respectively (Figure 5c; and Table S1, Supporting Information). These results, which are statistically equivalent to those obtained for group G3, suggest that the pretargeting strategy does not have an effect on the accumulation and/or retention of the labeled particles in the tumor. Such counterintuitive results might be due to different reasons, including: i) fast internalization of the mAb, which would decrease the amount of mAb on the cell surface and hence the amount of mAb-TCO available to undergo the click reaction with the Tz-functionalized AuNP; and ii) slow clearance of the AuNPs after accumulation in the tumor, due to fast cell internalization or limited drainage from the tumor tissue.

In order to gain further insight into the reasons behind the lack of significant differences between groups, we decided to carry out additional in vitro experiments. First, we investigated the cell internalization capacity of the nanoparticles in BT-474 cells. To perform the studies, the multifunctionalized AuNPs were fluorophore-labeled with TCO-Cy3 and incubated overnight with BT-474 cells. To visualize colocalization between

Figure 4. Representative image of agarose gel electrophoresis proving occurrence of click reaction. Lanes 1, 2, 6, and 7 present each component solo; lanes 3 and 5 are controls in which only one of the components is functionalized for the click reaction, and hence the click reaction is not occurring. The merged signal in lane 4 proves the click reaction between the two fully functionalized components, the mAb-TCO and the multifunctionalized AuNPs.
the AuNPs and the lysosomes, the latter were stained with LysoTracker (deep red). The obtained images show a clear colocalization between the AuNPs and the lysosomes (Pearson’s coefficient: $r = 0.648$; Manders’ coefficients: $M1 = 0.986$, $M2 = 0.972$), confirming internalization after overnight incubation (Figure 6a). For most drug delivery agents in cancer therapy, cell internalization favors treatment efficacy, as the drug is usually more efficacious when delivered into the cell. In the particular case of BNCT, cell internalization is not strictly required, and the location of the boron-rich compound in the vicinity of cancer cells should be sufficient for the lithium and alpha particles to induce local damage. However, if the boron-rich compounds are located close to the cell nucleus, the amount of boron required to achieve equivalent therapeutic efficacy is lower. Hence, cell internalization of the NPs can be perceived as a positive result. Still, in our particular case, using a pretargeting strategy, the situation is different. In pretargeting, the NPs should initially accumulate in the tumor due to the EPR effect. After entering the tumor tissue, NPs should react with the mAb-TCO present at the cell surface, resulting in enhanced retention and/or internalization. Such enhanced tumor retention should be relevant only when, in the absence of preinjection of mAb-TCO, the NPs are cleared from the tumor before internalizing. This, apparently, is not happening with the gold nanoparticles used in the current study, leading to equivalent results for the three groups.

The second reason behind the lack of differences in our study could be the fast internalization of the mAb-TCO into cells. This is not expected to happen, as recently published results suggest that $\approx 50\%$ of the mAb-TCO is not internalized in vivo at $t = 24\ h$ after administration.\cite{12} In any case, we decided to conduct further in vitro experiments to evaluate the pretargeting strategy between TCO-Trastuzumab and multifunctionalized AuNPs in cells. Therefore, the AuNPs were fluorophore-labeled with Cy3-NHS ester, which readily reacts with the PEG-amine functionalities of the particles. BT-474 cells were seeded in an

![Figure 5. a) Representative PET images (maximum intensity projections, coronal views) obtained at different time points after intravenous administration of $^{64}$CuAuNPs (group G3). All images have been coregistered with representative CT slices for localization of the radioactive signal; b) accumulation of multifunctionalized $^{64}$CuAuNP in different organs at different time points after intravenous administration, analyzed by PET imaging, for groups G1, G2, and G3. Values are expressed as mean ± standard error mean; $n = 4$ for groups G1 and G3; $n = 3$ for group G2; c) tumor uptake of $^{64}$CuAuNP in different experimental groups at different time points after administration. Values are expressed as mean ± standard error mean; $n = 4$ for groups G1 and G3; $n = 3$ for group G2; d) PET images (slices, axial view) showing local accumulation of the $^{64}$CuAuNP (group G3) in the tumor at different times after administration. PET images have been coregistered with the corresponding CT slice for anatomical localization.](image-url)
8-well plate (3 × 10^4 cells per well) and allowed to adhere overnight. Three different experimental groups were chosen and studied in parallel to enable comparison: i) Trastuzumab-TCO + fully functionalized, Cy3-fluorophore labeled AuNPs (pretargeting); ii) Trastuzumab nonfunctionalized + fully functionalized, Cy3-fluorophore labeled AuNPs (control 1); and iii) fully functionalized, Cy3-fluorophore labeled AuNPs (no mAb; control 2). The mAb for group i) and ii) was incubated for 30 min at 37 °C. The media with unbound mAb was removed, the Cy3-fluorophore labeled AuNPs added to all groups and incubated for 10 or 30 min (see Figure 6b for experimental set-up). The images of the different experimental groups, confirmed by the calculation of Pearson’s and Manders’ colocalization parameters (see Table S2, Supporting Information), showed very similar cellular uptake of the fluorophore labeled AuNPs, but with an increased signal after 30 min incubation compared to the 10 min incubation (Figure 6c). We expected significant differences in the pretargeting experiment after 10 min incubation of the AuNPs. If the TCO functionalized Trastuzumab was available on the cell membrane and if the click reaction occurred, an intense signal on the cell membrane should have been visible, which should decrease with longer incubation time due to internalization of the receptor-mAb-AuNP complex.[21] However, after 10 min incubation of the AuNPs, the fluorophore signal was low in all three cases and spread over the cell, colocalizing with the lysosomes, suggesting that internalization of the AuNP took place already. Hence, these results strongly suggest that the click reaction with Trastuzumab available at the cell surface and direct internalization of the AuNPs compete. However, the fast internalization of the AuNPs seems to be the main driving force for tumor retention. These results suggest that NPs with either lower cell internalization capacity or faster clearance from the tumor tissue are required in order to take advantage of the pretargeting strategy. In our case, faster clearance might be achieved by decreasing hydrodynamic diameter. This should also contribute to decrease the accumulation in organs with high presence of the MPS, such as the liver.

3. Conclusions

In this work, we report on the synthesis, characterization and evaluation of spherical gold nanoparticles (AuNPs) loaded with the boron cluster COSAN and functionalized with tetrazine, to enable the click reaction with TCO-Trastuzumab and hence suitable for the pretargeting approach. Small-sized AuNPs with core diameter 4.1 ±1.5 nm were successfully synthesized, multifunctionalized and characterized. Stability of the functionalization and biocompatibility was verified in vitro. Incorporation of the positron emitter ⁶⁴Cu was executed.

Figure 6. a) Representative image by live cell fluorescence microscopy of multifunctionalized AuNPs after overnight incubation in BT-474 cells. Green: AuNPs, fluorophore-labeled with TCO-Cy3; red: Lysosomes, stained with LysoTracker (deep red); blue: nucleus, stained with Hoechst 33 342. Merged signals of different fluorophores appear in yellow; b) 8-well plate with experimental set-up for pretargeting gold nanoparticles in vitro; c) representative images by fluorescence microscopy evaluating pretargeting AuNPs on BT-474 cells. As controls, incubation of AuNP alone (iii) and with nonfunctionalized Trastuzumab (ii) were performed. After 30 min incubation of mAb, incubation of AuNPs followed for either 10 min (left) or 30 min (right). Nucleus stained with Hoechst 33 342 (blue), AuNPs with Cy3-NHS (green), lysosomes with LysoTracker (deep red). Merged signals of fluorophores appear in yellow.
Chemistry and Radiochemistry—Conjugation of TCO-NHS to mAb: The monoclonal antibody (mAb) Trastuzumab (21 mg mL⁻¹, 2 mg) was diluted with PBS (phosphate-buffered saline, 10 × 10⁻³ M, pH 7.4) to a concentration of 3.0 mg mL⁻¹. The pH was adjusted to 8.6–9.1 with 0.1 M Na₂CO₃. Trans-Cyclooctene (TCO-NHS, 20 × 10⁻³ M in dimethylsulfoxide (DMSO), 26 μL, 50–55 eq.) was added. After incubation (120 min, room temperature) nonreacted TCO-NHS was removed by spin filtration (100 kDa, 16 128 g) and the conjugated mAb washed three times with PBS (pH 7.4). After recovering the mAb from the filter with PBS its concentration was determined by NanoDrop, using the default extinction coefficient for immunoglobulin type mAbs: ε(mAb) = 210 L (mmol × cm⁻¹⁻¹).

TCO Conjugation—Photometric Titration Analysis: To an aliquot of the TCO-conjugated mAb (0.05 mg; 0.3 nmol) was added 6-methyl-tetrazine-sulfo-Cy3 (mTzCy3, 1 mg mL⁻¹ in DMSO; 1:6 with respect to the initial amount of TCO-NHS). After 5 min incubation at room temperature, the fluorophore-labeled mAb was purified by spin filtration (100 kDa, 16 128 g) and washed four times with PBS. After recovering the mAb/TCO/mTzCy3 from the filter with PBS, the concentrations of mAb and mTzCy3 were determined by NanoDrop, using the extinction coefficients: ε(mAb) = 210 L (mmol × cm⁻¹⁻¹), NanoDrop default for immunoglobulin type mAbs; and ε(mTzCy3) = 151 L (mmol × cm⁻¹⁻¹), taken from datasheet of Jena Bioscience. The experiments were performed in duplicates.

Synthesis of AuNPs: For the synthesis of AuNPs the protocol reported by Zhao et al. was used,[20] with minor modifications. In brief, ultrapure water (0.9 mL) was mixed with aqueous HAuCl₄ (10 μL, 10 × 10⁻³ M; 0.4 mg, 1 eq.), aqueous CuCl₂ (10 μL, 10 × 10⁻³ M; 0.1 mg, 1 eq.), and aqueous NH₂-PEG₅-SH (25 μL, 10 × 10⁻³ M; 2.5 mg, 25 eq.) in a 10 mL glass vial. Under vigorous stirring, freshly prepared aqueous NaBH₄ solution (100 μL, 40 × 10⁻³ M; 0.2 mg, 4 eq.) was added dropwise. After 2 min, the stirring was stopped and the particles were allowed to settle for 2 h at room temperature. Purification was performed by spin filtration (30 kDa, 16 128 g) including 3 washings with PBS (pH 7.4) and the particles were recovered from the filter in PBS (400 μL). Subsequently, COSAN-SH solution in ethanol (13 μL, 20 × 10⁻³ M; 0.1 mg, 0.25 eq) was added and the mixture was incubated for 1 h at room temperature. After purification via spin filter as described above, the pH of the suspension was adjusted to 8–9 with 0.1 M NaOH and Tz-PEG₅-NHS (25 μL, 20 × 10⁻³ M in DMSO; 0.3 mg, 0.3 eq) was added. After 1 h incubation at room temperature the particles were purified by spin filtration (30 kDa, 16 128 g), washed three times with PBS and finally recovered in 400 μL PBS. In all cases, UV–VIS analysis was performed after each washing, to ensure that the ratio between the intensity of the bands corresponding to the plasmon and the ligand (i.e., COSAN or tetrazine) remained constant, ensuring thus the absence of free ligand. Characterization of the final particles was carried out using UV–VIS spectrophotometry, DLS, zeta-potential, ICP-MS, and TEM.

Fluorophore-Labeling of AuNPs with TCO-Cy3: The multifunctionalized AuNPs (450 μL in PBS, 0.2 mg mL⁻¹) were incubated with 3 μL TCO-Cy3 (10 × 10⁻³ M in DMSO) for 5 min at room temperature. The particles were purified by spin filtration (30 kDa) and recovered in PBS. The labeling was confirmed by UV–VIS spectra.

Fluorophore-Labeling of AuNP with Cy3-NHS: The multifunctionalized AuNPs (450 μL in PBS, 0.2 mg mL⁻¹) were adjusted with 0.1 M NaOH to pH 8.6–8.9 and Cy3-NHS (5 μL, 1 mg mL⁻¹ in DMSO) was added. After 1 h incubation at room temperature particles were purified by spin filtration (30 kDa) and recovered in PBS. The labeling was confirmed by UV–VIS spectrophotometry.

Gel Electrophoresis: A 1.5% agarose gel was prepared following standard protocol. In brief, agarose (1.5 g) was suspended in Tris-Borate-EDTA (TBE) buffer (100 mL) and heated until it almost reached its boiling point. The solution was poured in a gel chamber, the comb placed and let settle to polymerize. Samples were prepared in 20 μL aqueous batches, including 5 μL loading buffer. Samples (10 μL each) were loaded on the gel and run for ~1 h at 100 V. Visualization of the antibody on the gel was achieved by incubation in coomassie blue (1 h), followed by 2 days in destaining solution to reduce the background staining.

4. Experimental Section

Reagents: All reagents were of analytical grade and obtained from Sigma-Aldrich unless otherwise stated. Ultrapure water (resistivity 18.2 MΩ cm at 25 °C) was obtained from a Milli-Q A10 Gradient equipment (Millipore). Cy3-NHS was purchased from BroadPharm and Cy3-TCO from AAT Bioquest. The antibody Trastuzumab was purchased from S.A. (Spain). [¹²⁵I]CuCl₂ (in 0.1 M HCl) was produced in house with an IBA Cyclone 18/9 cyclotron using a (p, n) reaction on a gold disk, electroplated with enriched nickel-64 and the high boron content (close to 20% with respect to gold) position small-sized gold NPs as drug carriers with potential BNCT applications. However, the implementation of a successful pre-targeting strategy may require faster-cleared particles to maintain tumor uptake while minimizing accumulation in major organs such as the liver.
Synthesis of Radiolabeled AuNPs: The synthesis of ⁶⁴Cu-labeled AuNPs was carried out following a previously reported method, with minor modifications.[⁷] In brief, ultrapure water (0.9 mL) was mixed with aqueous HAuCl₄ (10 µL, 10 × 10⁻³ M) and different dilutions of 0.5 mL BT-474 cell suspensions (1 × 10⁶ cells in media). After 1 h incubation at room temperature the particles were purified by spin filtration and recovered in 400 µL PBS.

Radiolabeling efficiency and radiochemical purity were monitored by instant thin layer chromatography (iTLC) using 20 × 10⁻³ M citric acid + 60 × 10⁻³ M EDTA as the mobile phase; Rf(AuNP) = 0, Rf⁶⁴Cu-EDTA = 0.5. Subsequently, COSAN-SH solution in ethanol (13 µL, 20 × 10⁻³ M; 0.1 mg, 0.25 eq.) was added and the mixture was incubated for 1 h at room temperature. After purification via spin filter as described above, the pH of the AuNP suspension was adjusted to 7.8–8.8 with 0.1 M NaOH and Tz-PEG₂₉-NHS (25 µL, 20 × 10⁻³ M in DMEM; 0.3 mg, 0.5 eq.) was added. After 1 h incubation at room temperature the particles were purified by spin filtration and recovered in 400 µL PBS.

In Vitro Studies–Cells: BT-474 cells were cultured in Dulbecco’s modified Eagle’s medium ( Gibco), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, at 37 °C with 5% CO₂ in a humid atmosphere. Cells were confirmed to be free of mycoplasma contamination using the MycoAlert detection kit (Lonza).

Lindmo Assay: The assay was performed using the protocol of Lindmo.⁹ The binding assay for Trastuzumab was set up using one concentration of mAb (15 mg mL⁻¹) and different dilutions of 0.5 mL BT-474 cells (1 × 10⁶ cells mL⁻¹) in triplicates. The lowest concentration was prepared twice, the second set serving as nonspecific binding control (containing 2 µL of 5 mg mL⁻¹ nonlabeled mAb). To each cell dilution, radiolabeled Trastuzumab (0.5 mL) was added and incubated in a head over head rotator at 4 °C overnight. The cell suspensions were centrifuged, the supernatant (500 µL) separated and all samples (pellets and supernatants) measured in a gamma-counter. The activity measured from the supernatants was subtracted from the pellet activity to calculate the immuno reactive fraction.

Flow Cytometry Study with Trastuzumab-TCO-mTzCy₃: TCO conjugated Trastuzumab was fluorescence-labeled with 6-methyl-tetrazine-sulfo-Cy₃ (mTzCy₃) via click reaction. BT-474 cells were seeded in a 24-well plate (5 × 10⁵ cells per well) and incubated overnight to adhere (37 °C, 5% CO₂). The media was removed and 0.5 mL fresh media was added and incubated in a humid atmosphere. Cells were confirmed to be free of mycoplasma contamination using the MycoAlert detection kit (Lonza).

PET Imaging Studies: To obtain the BT-474 breast cancer xenograft model, NOD/SCID mice were implanted with 0.72 mg, 60-day release, 17β-estradiol pellets (E2-M/60, Belma Technologies). One day later, 10⁷ BT-474 cells were subcutaneously inoculated using a 25-gauge needle in the back of each mouse. Cells were diluted in Matrigel matrix (Corning) at a final volume of 0.2 mL (1:1 ratio between cell and Matrigel). Tumor growth was monitored by serially measuring the diameters with an electronic caliper and calculating the tumor volumes by the formula: volume = width² × length / 2. One tumor reached approximately a volume of 300 mm³, animals were randomly divided in three groups (G1 and G3: n = 4, G2: n = 3). For the pretargeting group (G1) TCO functionalized Trastuzumab (~100 µg, 1 µg mL⁻¹) was intravenously injected via tail vein. The control group (G2) received an intravenous injection with the same amount of nonfunctionalized Trastuzumab.

Cytotoxicity Studies of AuNPs: To determine cell viability, BT-474 human breast cancer cells were incubated with multifunctionalized AuNPs over 24, 48, and 72 h. Cells were seeded (3 × 10⁴ cells per well, 100 µL per well, 96-well plate), allowed to adhere overnight in complete media and maintained in a humid atmosphere at 37 °C and 5% CO₂. Then, media was removed and cells were left untreated (control) or incubated with the AuNP-containing formulations, diluted accordingly in media. After the desired time, cell supernatant was removed and MTT reagent (100 µL per well; Roche), diluted in the corresponding media to the final concentration of 0.25 mg mL⁻¹, was added. After 1 h incubation at 37 °C and 5% CO₂, the excess reagent was removed and formazan crystals were solubilized by adding DMSO (200 µL per well). The optical density of each well was measured in a TECAN Genios Pro 96/384 microplate reader at 550 nm. Data were represented as the percentage of cell survival (mean ± standard error mean, n = 6) compared to controls well.
of injected dose per cubic centimeter (%ID cm⁻¹; mean ± standard deviation).

Statistical Analysis: Statistical significance of differences in between methods was calculated using 2-way ANOVA followed by Tukey’s test for multiple comparisons. Differences were concluded significant for P values < 0.05. Statistical tests were performed using GraphPad Prism 7.03 (GraphPad Software, La Jolla, CA).

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest
The authors declare no conflict of interest.

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