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In vitro binding kinetics study of gold nanoparticles functionalized with ^{68}Ga-DOTA conjugated peptides

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Abstract In this study we tested functionalized AuNPs as an improved drug delivery tool in pancreatic and colon cancer cell lines AR42J and HT-29, using radioimmunoassay method. ^{68}Ga radiolabeled DOTA-Tyr(3)-octreotide, DOTA-NaI(3)-octreotide and DOTA-NT peptides were linked on nanoparticles surface, aiming to increase the peptide density at the tumor site and thus interaction probability, resulting in higher photon signal of ^{68}Ga radioisotope used in positron emission tomography. In vitro ligand-receptor binding evaluation of the radiolabeled compound has showed increased retention of the positron emitter radionuclide ^{68}Ga in the presence of the gold nanoparticles.

Keywords Gold nanoparticles · Cancer diagnostic · Radiolabeled peptides · Octreotide · Neurotensin

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Introduction

^{68}Ga is a short-lived ($T_{1/2}$ = 67.83 min) radioisotope obtained after ^{68}Ge/^{68}Ga generator elution, decaying 89 % through positron emission and 11.1 % by electron capture into ^{68}Zn. Although it is available since over 30 years, its clinical research for positron emission tomography (PET) application, has increased only in the last years, after a new class of target-specific radiopharmaceuticals have emerged. ^{68}Ge parent radionuclide has a physical half live of 270.9 days, which enables the use of generator for almost one year at lower costs compared to a cyclotron facility. Despite the higher energy of the positron emission of ^{68}Ga (maximum energy = 1.9 MeV) compared to that of the widely used ^{18}F (maximum energy = 0.6 MeV), ^{68}Ga provides the possibility of PET examinations to the medical centers that do not own an accelerator, or are located at long distance from the [^{18}F]FDG distribution area. ^{68}Ga-based radiopharmaceuticals are generally composed of a chelator agent that bind the radioisotope in complex, and a biologically active targeting moiety, like peptide, hormone or antibody, that bind with high affinity to specific cell surface receptors from the human body.

Gold nanoparticles functionalized with radiolabeled peptides have tremendous growth in pharmaceutical field, based on NPs ability to pass the cells membrane by receptor-mediated endocytosis [1]. Nanoparticles binding to biomolecules depend upon ligand containing amine, carboxyl or thiol groups that can be used to covalently attach biomolecules to nanoparticles surface [2, 3]. Coupling methods, often may lead to the degradation or inactivation of biological active sequence of attached molecules. For this purpose, the above mentioned groups should be positioned in terminal position of the molecule or be functionalized on nanoparticles surface [4]. An

example of gold nanoparticles functionalized with somatostatin analog-octreotide is illustrated in Fig. 1, where amino terminal group of Tyr(3)-octreotide (TOC) serves as linker between them. Although in vivo application of gold nanoparticles, thiol terminated groups are the most desired one, because they have the highest affinity to noble metal surfaces, amino groups can be used as linker between NPs and peptides but for a limited time.

Somatostatin analog TOC is one of the latest introduced ^{68}Ga labeled peptide in the European Pharmacopoeia, as ^{68}Ga-Edotreotide injection. This cyclic peptide, is targeting with high affinity somatostatin receptor SSTR2 expressed on neuroendocrine tumors. Because moreover, the majority of human tumors do express more than one receptor subtype, other somatostatin analogs have been synthesized, like NaI(3)-octreotide (NOC) who bind with high affinity to SSTR2, SSTR3 and SSTR5 receptors [5].

In the present study, we have successfully synthesized and functionalized gold nanoparticles with three DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) conjugated peptides: neurotensin, NOC and TOC. All peptides were previously radiolabeled with short-lived ^{68}Ga radioisotope obtained from ^{68}Ge/^{68}Ga generator. The radiolabeled complexes were analyzed in terms of radiochemical purity and stability. Nanoparticles physicochemical characterization was performed for determination of hydrodynamic diameter, colloidal stability and maximum absorption corresponding wavelength. Also the successful functionalization of AuNPs was followed through red shift in the absorption spectra of the nanoparticles. Later on, the radiolabeled preparations were incubated on colon cancer cells HT-29 and exocrine pancreatic tumor cells AR42J.

The aim of the study was to evaluate the biological effect of AuNPs upon radioisotope uptake and retention using radioimmunoassay method of LigandTracer Yellow instrument.

Experimental

Synthesis of gold nanoparticles

Nanoparticles preparation was carried out using Turkevich method [6, 7]. 1.25 mL of $HAuCl_4 \times 3H_2O$ solution (20 mM) (Carl Roth GmbH + Co. KG) were dissolved in 1 L distilled water at 80 °C, while kept under vigorous stirring (Ceramic Hot Plate Stirrer—HSC from VELP Scientifica). As reducing agent trisodium citrate dihydrate (16.6 g/L) (Carl Roth GmbH + Co. KG) was added, continuing the stirring process and keeping the temperature constant for at least 20 min., until a deep red stable dispersion was obtained. Sodium citrate also stabilizes the AuNPs by forming a negatively charged double layer [8].

Nanoparticles characterization

Nanoparticles formulation was analyzed for morphology, size distribution and average particle hydrodynamic diameter using dynamic light scattering (DLS) and transmission electron microscopy (TEM) methods. In order to assess nanoparticles formulation stability, electrostatic potential near the NP surface called zeta (ζ) potential was evaluated through PALS (phase analysis light scattering) technique provided by Malvern Nanosizer 90S system.

For further characterization, UV–Vis measurement was performed on Specord 210-Analytic Jena AG spectrophotometer, using 0.75 mL quartz cuvette.

Quality control and peptides radiolabeling with ^{68}Ga radioisotope

DOTA-NaI(3)-octreotide (DOTA-NOC), DOTA-Tyr(3)-octreotide (DOTA-TOC) and DOTA-neurotensin (DOTA-NT) peptides purchased from piChem, Austria, were labeled with short lived positron emitter ^{68}Ga using organic column type ^{68}Ge/^{68}Ga generator (ITG Isotopen Technologien München

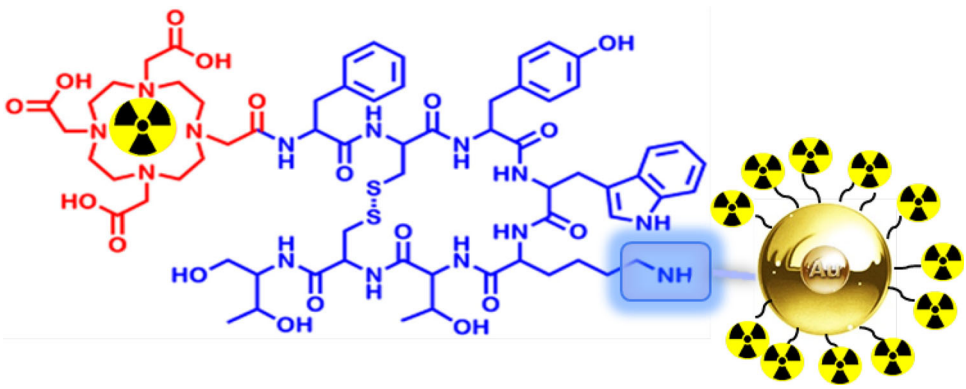

Fig. 1 Surface functionalization of gold nanoparticles with radiolabeled somatostatin analog Tyr(3)-octreotide

AG, Germany) and automated labeling module Galigand GAL-102. For radiolabeling process $^{68}Ge/^{68}Ga$ generator was eluted with 4 mL HCl 0.05 M obtained by dilution of 30 % HCl ultrapure (Merck KGaA) with ultrapure water. From the total eluate volume (4 mL), the first and the last fractions (a total of 2 mL) containing radioactive and metalic impurities and low amount of ^{68}Ga were sent to the waste collecting vial. The midle fraction of the eluate consisting of 2 mL $^{68}GaCl_3$, which concentrates the most of the ^{68}Ga amount, was then collected in the reaction vial. The available radioactivity for labeling processes was about 630 MBq for DOTA-NOC and between 410 and 430 MBq for DOTA-NT and DOTA-TOC. Depending of the biomolecule used for labeling, ~22 nmol of DOTA-NOC, 35 nmol DOTA-TOC and 24 nmol DOTA-NT were added, previously mixed with 1 M ammonium acetate buffer solution (Sigma Aldrich) to avoid chemical degradation of the peptide [9]. In order to bind ^{68}Ga using DOTA as chelating agent, a temperature of 95 °C was used [10]. The reaction time required for labeling is 5 min, after which the reaction mixture was passed through Phenomenex Strata-X SPE cartridge in order to separate the biomolecule from the free ^{68}Ga and other impurities which pass through the column and go to waste. The column requires previous conditioning with 1 mL ethanol (Chimreactiv, Romania) followed by 1 mL ultrapure water. The peptide is rinsed on the column with a mixture made of 950 µL ultrapure water and 50 µL ethanol. The radiolabeled peptide is then recovered by rinsing the cartridge with 1 mL of ethanol and transferred to a 95 °C preheated vial for ethanol dry evaporation step. In order to ensure physiological pH of the final radiolabeled complex, 1 mL of physiological saline solution is added (0.9 % NaCl solution, B Braun Melsungen).

After labeling, the radiochemical purity was assessed through HPLC using C-18 column, Nucleosil, as stationary phase and acetonitrile and water acidified with 0.1 % TFA as mobile phases [11]. The radiochemical stability of the radioligand complex was evaluated by HPLC in 0.9 % physiological saline solution at 1, 2, 3 and 4 h after the end of the synthesis.

Conjugation of ^{68}Ga-DOTA-TOC, ^{68}Ga-DOTA-NT and ^{68}Ga-DOTA-NOC to AuNPs

For ^{68}Ga-DOTA-peptide-AuNP complex preparation, 1 mL of nanoparticles suspension (having a concentration of 30 mg/L) was mixed with 500 µL ^{68}Ga-DOTA-NOC/TOC/NT (containing 17.5 nmol of TOC, 11.25 nmol of NOC and 12 nmol of NT) and the mixture was stirred for 15 min (450 rot/min.) at room temperature. As we observed from previous experiments, exceeding 15 min stirring can cause nanoparticles irreversible aggregation. No further purification was performed.

In order to assess the nanoparticles payload with the radiolabeled peptides based on gold nanoparticles wavelength shift, additional UV–Vis evaluation was necessary.

Cell culture

Cell lines used in the experiments include the amphicrine pancreatic tumor cell line AR42J (Biochrom GmbH, Berlin, Germany) and the colon cancer cell line HT-29 (Biochrom GmbH, Berlin, Germany). All cells were passaged twice weekly in Ham's F-12 medium for AR42 cells, supplemented with 20 % fetal bovine serum and 1 % L-glutamine. HT-29 cells were cultured in DMEM medium. All substances were purchased from Biochrom GmbH, Berlin, Germany. The cells were grown at 37 °C in humidified air containing 5 % CO_2.

Approximately 5×10^5 pancreatic tumor cells and 4×10^5 colon tumor cells were seeded 24 h prior to use in a local area of the 87 mm diameter petri dish (Nunclon™ Delta Surface 150,350, Thermo Fisher Scientific). In order to use the LigandTracer technology, the dishes were placed slightly tilted during the cells attachment period.

In vitro binding kinetics study

The goal of this experiment was to investigate by comparison the binding of radiolabeled conjugates and functionalized gold nanoparticles on tumor cells. For this purpose we assessed the cell-associated radioactivity as a function of the incubation time using LigandTracer technology (Ridgeview Instruments AB, Sweden) [12, 13].

Prior to each measurement the cell medium was changed with 1 mL of pure medium. The measurements were then performed according to the following steps, as illustrated in Fig. 2:

(a) One data point was acquired for background measurement in order to assess the final retention level of the radiolabeled complex.
(b) 3.5 nmol radiolabeled DOTA-TOC/NOC and 2.4 nmol of DOTA-NT were incubated until steady state conditions were reached. An average incubation time of 35 min has been considered the appropriate time to reach steady state [14]. Also because of the short physical half-life of ^{68}Ga radioisotope we limited the measurements to 1 h.
(c) After the equilibrium condition has been occurred the radioactive medium has been removed, followed by cell dish rinsing with 2 mL of DMEM/Ham's F-12 medium. After the rinsing procedure, the cells were supplied with 1 mL of cell medium.
(d) The final part of the kinetic assay consisted in retention evaluation for a variable time, until the

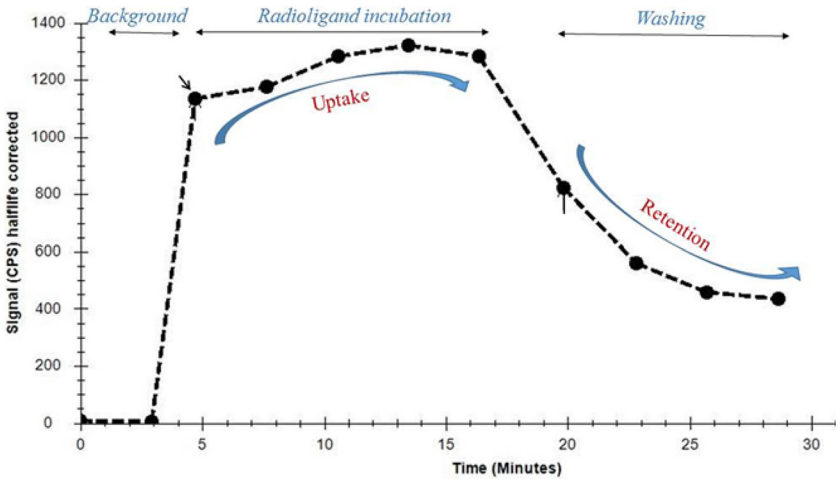

Fig. 2 Example of binding trace using LigandTracer instrument

retained radioactive complex activity has dropped up to a saturation level. This level reflects the actual amount of radioisotope restrained on the cell membrane or inside the tumor cells.

Same process has been repeated for gold nanoparticles functionalized with radiolabeled peptides.

Results and discussion

Nanoparticles characterization

The average particle hydrodynamic diameter determined using DLS was 42.57 ± 4 nm as depicted in Fig. 3, ζ potential indicated good colloidal stability with −41.5 ± 6 mV.

^{68}Ga radiolabeled peptides synthesis and quality control

Table 1 summarizes the radiolabeling yield obtained after the synthesis process, also the radiochemical purity evaluated through high performance liquid chromatography (HPLC) system. Radiolabeling yield was calculated dividing the final activity A_F of pure radiolabeled complex to the sum of activity A_C retained on SPE cartridge, activity A_{EW} of the resulted elution waste (the other 2 mL of $^{68}GaCl_3$ not used in the synthesis process) and reaction waste activity A_{RW} resulted during the synthesis process:

$$\eta = \frac{A_F}{A_c + A_{EW} + A_{RW}} \quad (1)$$

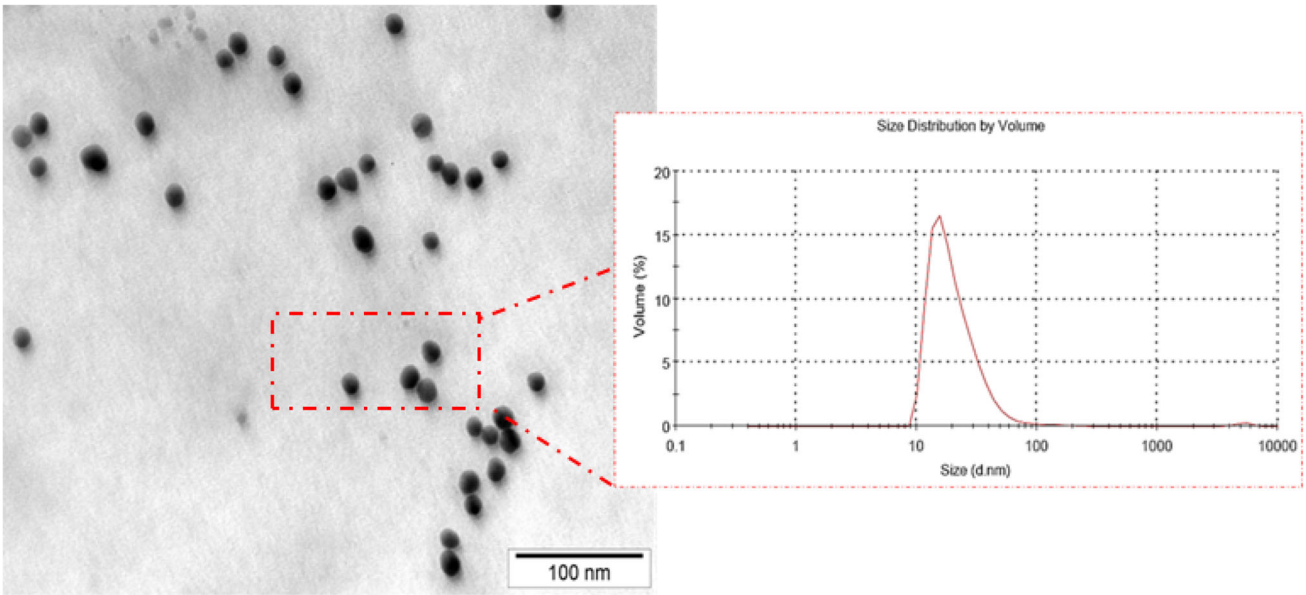

Fig. 3 Gold nanoparticles hydrodynamic diameter evaluation by TEM microscopy and DLS techniques

Table 1 Radiolabeling parameters and chemical purity

Labeled peptide	Amount of radiolabeled peptide (nmol)	Specific activity (MBq/nmol) (%)	Radiolabeling yield (%)	Radiochemical purity (%)
DOTA-Thr(3)-octreotide (TOC)	35	6.9 ± 22	62 ± 3	>98
DOTA-NaI(3)-octreotide (NOC)	22	12 ± 27	86 ± 2	>99
DOTA-neurotensin (NT)	24	14 ± 13	80 ± 6	100

High performance liquid chromatography analysis upon radiochemical purity of labeled conjugates indicated over 98 % chemical purity. As illustrated in Figs. 4, 5 and 6 elution peak of ^{68}Ga-DOTA-TOC appears after $t_R = 18.61$ min, $t_R = 18.37$ min for ^{68}Ga-DOTA-NT and $t_R = 22.77$ min, for ^{68}Ga-DOTA-NOC. 4 h post EOS (end of synthesis), the radiochemical purity maintained over 98 % which indicates the stability of the final products after passing four half-lives of the ^{68}Ga radioisotope, results which are complying with the standards of the European Pharmacopoeia for radiopharmaceuticals preparations.

Gold nanoparticles functionalization

The maximum optical absorbance of the plasmon band of bare gold NP was observed at $\lambda \sim 524$ nm, which correspond to the previous findings in literature [15]. Wavelength shift induced by changes in the refraction index and the surrounding dielectric medium, which occurred as consequence of the interaction between the peptide and the AuNP surface, was evaluated by UV–Vis optical absorption spectra (Fig. 7), showing 2 nm red shift for ^{68}Ga-DOTA-NT-AuNP, 3 nm for ^{68}Ga-DOTA-NOC-AuNP, respectively 5 nm for ^{68}Ga-DOTA-TOC-AuNP.

Uptake and retention assay

In vitro assay aimed to study two characteristics of the peptide-cell interaction process: cellular uptake and cellular retention.

The first part of the binding trace consists of uptake measurement, which corresponds to radiolabeled conjugates incubation time. The measurements results were decay corrected to the half-life of ^{68}Ga (67.83 min) and in order to compare the two measurements performed on every peptide (one for ^{68}Ga radiolabeled peptide and one for gold nanoparticles functionalized with same radiolabeled peptide) the final data were normalized.

The second part of the binding trace consists of retention evaluation by removing the hot medium from cells, rinsing the cell dish with fresh medium and resume the measurement in order to assess the amount of radioactivity detected due to peptide attachment to its specific receptor from the cell surface. All studies used 3.5 nmol of radiolabeled peptide, different radioligand volumes being needed to ensure this quantity. Parameters used in the uptake/retention process are summarized in Table 2.

The binding kinetics of both cellular uptake and retention of the somatostatin analog Tyr(3)-octreotide are presented in Fig. 8. As one can see the uptake slope of the radiolabeled complexes are almost the same, leading the

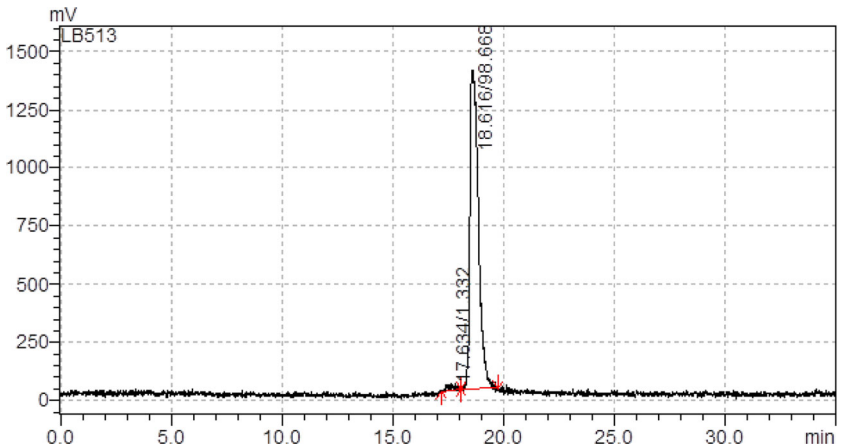

Fig. 4 HPLC chromatogram of final ^{68}Ga-DOTA-TOC complex

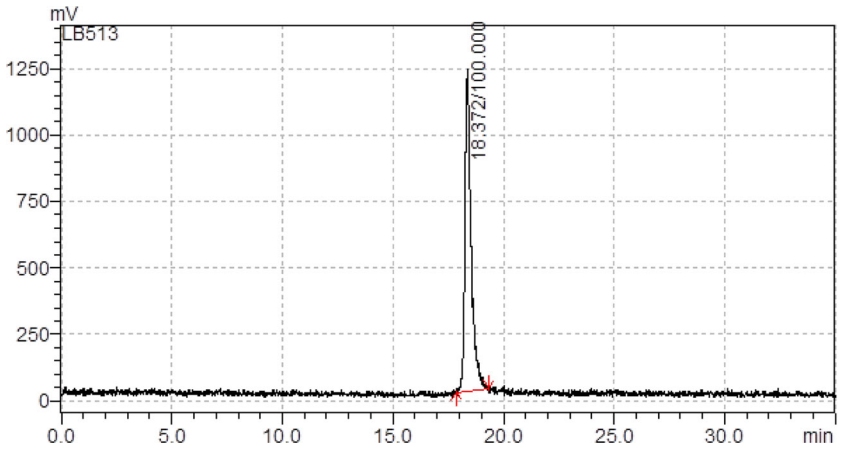

Fig. 5 HPLC chromatogram of final ^{68}Ga-DOTA-NT complex

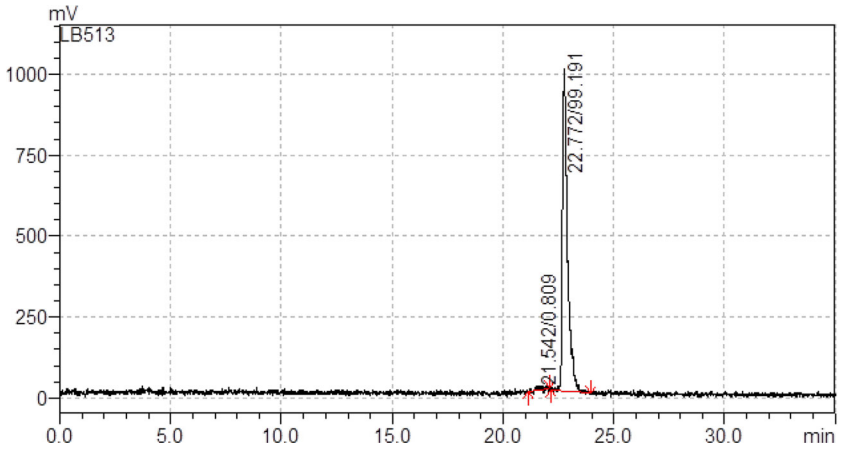

Fig. 6 HPLC chromatogram of final ^{68}Ga-DOTA-NOC complex

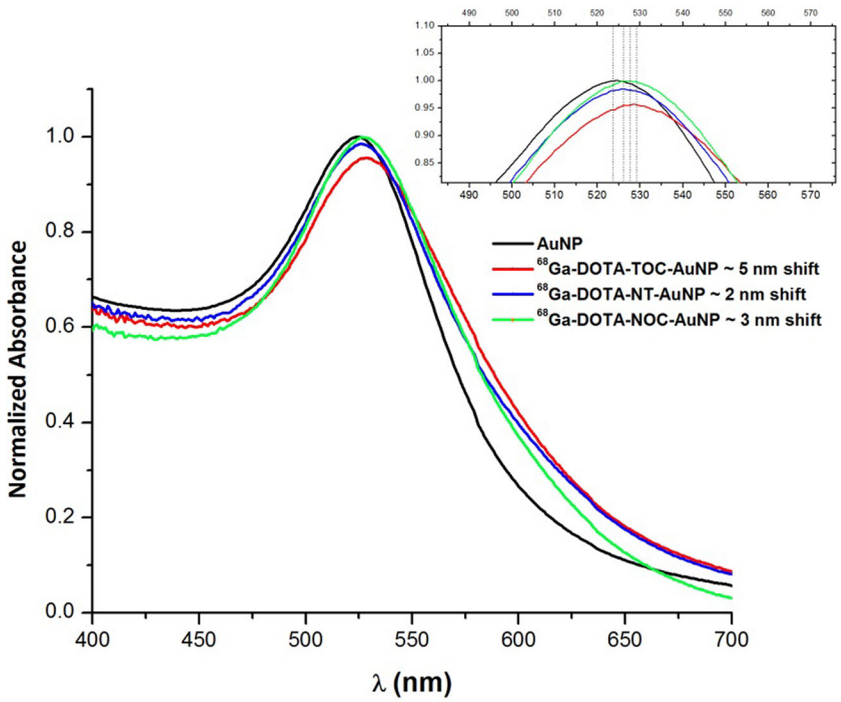

Fig. 7 Optical absorption spectra for AuNPs functionalization with ^{68}Ga-DOTA-TOC, ^{68}Ga-DOTA-NOC and ^{68}Ga-DOTA-NT

Table 2 The binding kinetic assay parameters

Radiolabeled complex	Amount of incubated peptide (nmol)	Radioligand volume (μL)	Number of cells in the dish/cell line	Incubation time (min)
^{68}Ga-DOTA-TOC	3.5	100	5×10^5 (HT-29)	20
^{68}Ga-DOTA-TOC-AuNP	3.5	300	5×10^5 (HT-29)	20
^{68}Ga-DOTA-NOC	3.5	156	5×10^5 (HT-29)	40
^{68}Ga-DOTA-NOC-AuNP	3.5	466	5×10^5 (HT-29)	40
^{68}Ga-DOTA-NT	3.5	146	4×10^5 (AR42J)	20
			5×10^5 (HT-29)	40
^{68}Ga-DOTA-NT-AuNP	3.5	438	4×10^5 (AR42J)	20
			5×10^5 (HT-29)	40

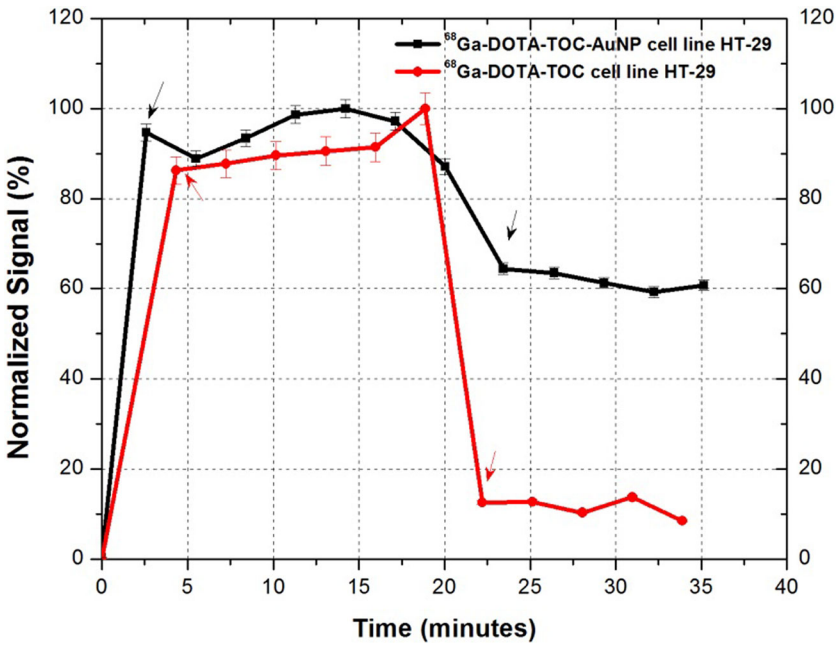

Fig. 8 The binding trace during incubation and after incubation of ^{68}Ga-DOTA-TOC-AuNP in comparison with ^{68}Ga-DOTA-TOC. Radioligand incubation time and hot medium change are indicated by *arrows*

conclusion that gold nanoparticles do not interfere in the uptake process of the radioisotope. In return, the retained radioactivity difference between the two conjugates is around 50 %, from 10 ± 3 % in the case of ^{68}Ga-DOTA-TOC up to 60 ± 2 % ^{68}Ga-DOTA-TOC-AuNP.

As in the case of TOC, NOC peptide complexes have the same trend line for uptake but functionalized gold nanoparticles have a 35 % elevated radioligand retention compared to ^{68}Ga-DOTA-NOC, as depicted in Fig. 9.

At the beginning, for radiolabeled neurotensin conjugates we performed the assay on HT-29 cell line, but the results showed only 10 % improved retention so we also evaluate the radioisotope accumulation on AR42J cell line (Fig. 10). For the second tumor cell line tested, the same retention level could be observed (Fig. 11). Because neurotensin have multiple NH_2 groups in its structure, not just in the terminal position, we assumed that there is a possibility that both linker and acceptor implicated in AuNP-neurotensin interaction are immobilized on the surface of different nanoparticles and induce their assembly [16].

Conclusions

In summary this in vitro binding kinetics study evaluated the ability of gold nanoparticles to increase the available ^{68}Ga positron emitter radioisotope inside tumor cells, using together AuNPs properties to pass cells membrane by

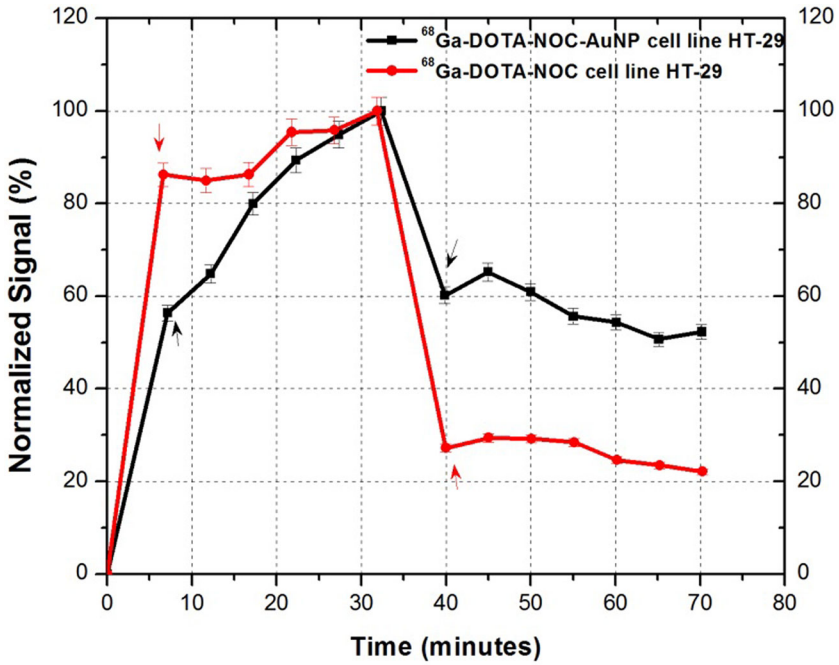

Fig. 9 The binding trace during incubation and after incubation of ^{68}Ga-DOTA-NOC-AuNP in comparison with ^{68}Ga-DOTA-NOC. Radioligand incubation time and hot medium change are indicated by *arrows*

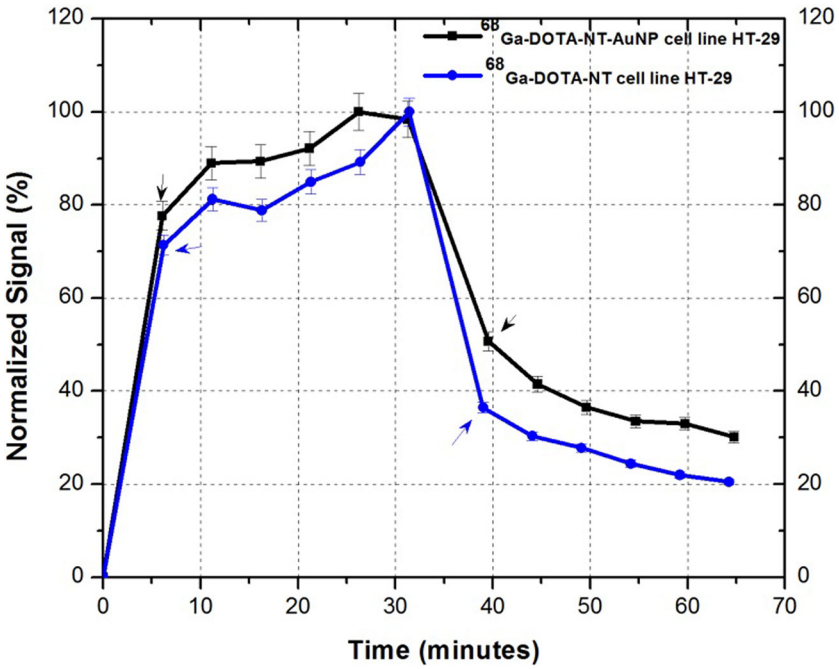

Fig. 10 The binding trace during incubation and after incubation of ^{68}Ga-DOTA-NT-AuNP in comparisonn with ^{68}Ga-DOTA-NT, on HT29 cell line. Radioligand incubation time and hot medium change are indicated by *arrows*

endocytosis, and radiolabeled peptides specific tumor targeting properties.

Gold nanoparticles functionalized with ^{68}Ga-DOTA-NOC and ^{68}Ga-DOTA-TOC showed over 60 % radionuclide retention after hot medium extraction, with an average of 35 %, respectively 50 % improved retention in comparison with ^{68}Ga-DOTA-NOC and ^{68}Ga-DOTA-TOC conjugates. In contrast, neurotensin functionalized AuNPs showed 10 % slightly elevated retention in comparison with ^{68}Ga-DOTA-NT.

These results are attributed to receptors mediated uptake of the nanoparticles inside the tumor, and their possibility to bind several peptides on their surface, increasing the available radioisotope inside the tumor cells. We anticipate that in vivo application of these functionalized AuNPs, with proper coating, might improve the quality of the PET images.

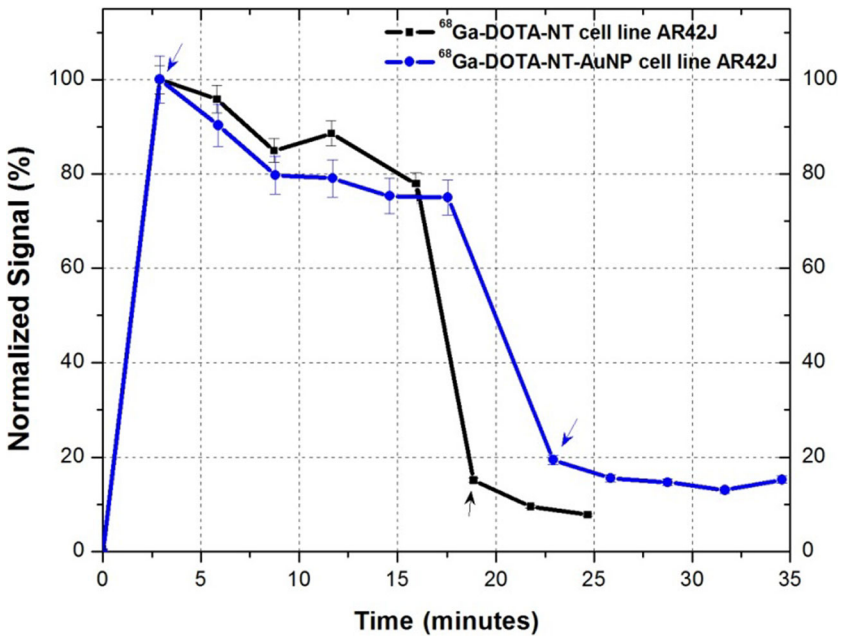

Fig. 11 The binding trace during incubation and after incubation of ^{68}Ga-DOTA-NT-AuNP in comparison with ^{68}Ga-DOTA-NT, on AR42J cell line. Radioligand incubation time and hot medium change are indicated by *arrows*

Further studies need to be performed upon these functionalized nanoparticles toxicity and also their possible interaction with peptides properties to bind on specific receptors site.

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