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RESEARCH PAPER

Octreotide Nanoparticles Showed Affinity for *In Vivo* MIA Paca-2 Inducted Pancreas Ductal Adenocarcinoma Mimicking Pancreatic Polypeptide-Secreting Tumor of the Distal Pancreas (PPoma)

Thaís Ligiéro Braga[1] • Suyene Rocha Pinto[1] • Sara Rhaissa Rezende dos Reis[1] • Filipe Leal Portilho[1] • Aline Oliveira da Silva de Barros[1] • Emerson Soares Bernardes[2] • Sofia Nascimento dos Santos[2] • Luciana Magalhães Rebelo Alencar[1,3] • Eduardo Ricci-Junior[4] • Ralph Santos-Oliveira[1,5]

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ABSTRACT

Purpose Pancreatic Polypeptide-secreting tumor of the distal pancreas (PPoma) is a rare, difficult and indolent type of cancer with a survival rate of 5-year in only 10% of all cases. The PPoma is classified as a neuroendocrine tumor (NET) not functioning that overexpresses SSTR 2 (somatostatin receptor subtype 2). Thus, in order to improve the diagnosis of this type of tumor, we developed nanoparticulate drug carriers based on poly-lactic acid (PLA) polymer loaded with octreotide and radiolabeled with Technetium-99 m (^{99m}Tc).

Methods PLA/PVA octreotide nanoparticles were developed by double-emulsion technique. These nanoparticles were characterized by Atomic Force Microscopy (AFM) and Dynamic Light Scattering (DLS) and radiolabeled with 99mTc by the direct via forming ^{99m}Tc-PLA/PVA octreotide nanoparticles. The safety of these nanosystems was evaluated by the MTT cell toxicity assay and their *in vivo* biodistribution was evaluated in xenografted inducted animals.

Results The results showed that a 189 nm sized nanoparticle were formed with a PDI of 0,097, corroborating the monodispersive behavior. These nanoparticles were successfully radiolabeled with 99mTc showing uptake by the inducted tumor. The MTT assay corroborated the safety of the nanosystem for the cells.

Conclusion The results support the use of this nanosystem (^{99m}Tc-PLA/PVA octreotide nanoparticles) as imaging agent for PPoma.

KEY WORDS imaging agent · nanoradiopharmaceuticals · octreotide · PPoma · radiopharmacy

ABBREVIATIONS

AFM	Atomic Force Microscopy
CO_2	Carbon Dioxide
DLS	Dynamic Light Scattering
DMEM	Dulbecco ModifiedEagle'sMinimalEssentialMedium
EDTA	Ethylenediaminetetraaceticacid
EE	Entrapment Efficiency
EPR	Enhanced Permeability and Retention
FBS	Fetal BovineSerum
HEPES	N-2 Hydroxyethyl Piperazine-N′-2 Sulfonic Acid Ethane
ID/organ	Dose per Organ
IPEN/ CNEN	*Instituto de Pesquisas Energéticas e Nucleares/ Comissão Nacional de Energia Nuclear*_Institute of Energy and Nuclear Research/National Nuclear Energy Commission
μCi	Microcurie
MBq	Megabecquerel
MIA PACA-2	Cells from human pancreas carcinoma
MPS	Mononuclear Phagocytic System
MTT	3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide

✉ Ralph Santos-Oliveira
presidenciaradiofarmacia@gmail.com

[1] Brazilian Nuclear Energy Commission, Nuclear Engineering Institute, Rio de Janeiro, Brazil

[2] Centro de Radiofarmácia, Instituto de Pesquisas Energéticas e Nucleares, São Paulo, Brazil

[3] Department of Physics, Federal University of Maranhão, São Luís, Maranhão, Brazil

[4] Faculty of Pharmacy, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

[5] Laboratory of Radiopharmacy and Nano-radiopharmaceuticals, Zona Oeste State University, Rio de Janeiro, Brazil

$NaHCO_3$	Sodium Bicarbonate
NET	Neuroendocrine Tumor
PDI	Polydispersity Index
PLA	Polylactic Acid
PP	Pancreatic Polypeptide
PPoma	Pancreatic Polypeptide-secreting tumor of the distal pancreas
PVA	Poly(Vinyl Alcohol)
RPMI	Roswell Park Memorial Institute
SC	Subcutaneous
SD	Standard Deviation
$SnCl_2$	Stannous Chloride
SSTR 2	Somatostatin Receptor Subtype 2
^{99m}Tc	Technetium 99 metastable
UT	Untreated cells
W/O	Water/organic emulsion
W/O/W	Water/organic solvent/water emulsion

INTRODUCTION

The Pancreatic Polypeptide-secreting tumor of the distal pancreas (PPoma) is classified as a neuroendocrine tumor (NET) not functioning that overexpresses SSTR 2 (somatostatin receptor subtype 2). This type of cancer affects the right side of the organ (the head) where 90% of the PP cells (responsible to produce the pancreatic polypeptide hormone) are found (1–4). The PPoma is considered a rare tumor more prevalent in individuals between the 5th and 6th decade of life (2,5–9).

The PPoma cancer overexpress the pancreatic polypeptide (PP), which is composed of 36 amino acid peptide produced and secreted by PP cells primarily located in the Islets of Langerhans. Is important to notice that this pancreatic peptide is biologically inactive, being considered a clinically silent tumor. This fact, leads to a difficult diagnosis, which often occurs accidentally (2,5–11). Also the PPoma are generally unresectable and in 90% of the cases they are metastatic, with a survival rate of 5-year in only 10% of all cases (12). The high malignant potential of PPoma (90%) is mainly due to its slow and silent growth, reaching large dimensions at the time of diagnosis (5.9 cm) (2,9,13,14). The main risk factors related to PPoma are: high saturated fat intake; malignancy - associated impairment in glucose metabolism; thyroid dysfunction; alcohol consumption; smoking; medical conditions such as diabetes mellitus and environmental factors such as radiation. It is known that the risk for developing pancreatic cancer is 1 in 67 (2,3,15,16).

Octreotide is an octapeptide, developed as a drug, who acts as a potent octapeptide analog of somatostatin. The various actions of somatostatin are mediated through specific membrane receptors, especially the octreotide binds with high affinity to somatostatin receptor subtype 2 and subtype 5 and with moderate affinity to subtype 3. In the case of PPoma the most abundant receptor is the SSTR 2 (11,17,18).

Thus, considering that the PPoma is an aggressive and incurable tumor with a high lethal rate and without any specific tumor test, we developed, characterized and preclinically tested a ^{99m}Tc-PLA/PVA/Octreotide nanoparticles as a new nano-radiopharmaceutical for early and differential diagnosis of PPoma.

MATERIALS AND METHODS

Synthesis of Ocreteotide Nanoparticle

Nanoparticles were prepared by the double emulsification with solvent evaporation method, according to the protocol described by Patricio *et al.* (19). A aqueous solution of 0.6 mL of poly(vinyl alcohol) (PVA, 0.1%*w/v*, 85% hydrolysed; Sigma-Aldrich, USA) and 0.4 mL Octreotide (4% w/v; Norvatis) was prepared. This solution was poured into 50 mg of polylactic acid (PLA, 40–100 kDa, 0.15–0.25 viscosity, Sigma-Aldrich, USA) in 4 mL of dichloromethane and then emulsified by sonication (ultrasonic processor UP100H, 100 W, 30 kHz, Hielscher Ultrasonics, Teltow, Germany) for 2 min (55 W), producing a water/organic solvent (W/O) emulsion. This solution was then emulsified with 10 mL of PVA (1% *w/v*) solution by sonication for 2.5 min (55 W), producing a water/organic solvent/water (W/O/W) emulsion. Then, the organic solvent was removed by evaporation under a vacuum (1.5 h) (Rotavapor Water bath B-480, Büchi Labortechnik AG, CH, Switzerland) at 25°C at 60 rpm. The particles were recovered by centrifugation (Avanti™ J-25 High Performance Centrifuge, Beckman Instruments, Palo Alto, CA, USA) at 20,000 rpm for 20 min at 20°C to remove the excess PVA. This procedure resulted in PLA/PVA/Octreotide nanoparticles.

Dynamic Light Scattering

Size distribution, mean size and polydispersity index (PDI) of the PLA/PVA/Octreotide nanoparticles were determined by dynamic light scattering (DLS) using the equipment Zetasizer Nano ZS (Malvern Instruments, UK). Measurements were performed in triplicate at 25°C and the laser incidence angle in relation to the sample was 173° using a 12 mm^2 quartz cuvette. The mean ± standard deviation (SD) was assessed (https://iopscience.iop.org/article/10.1088/1361-6528/ab177c/meta).

Atomic Force Microscopy

The morphology and dimensions of the PLA/PVA/Octreotide nanoparticles were analyzed by atomic force microscopy (AFM) using a Multimode 8 (Bruker, Santa Barbara, CA, USA) coupled with software NanoScope Analysis 1.50 (Bruker, Santa Barbara, CA, USA), in tapping mode (intermittent

contact) for acquisition of topographic and phase images to investigate diameter, height and phase composition of particles. All the experiments were performed with cantilevers spring constant of 0.24 N/m and nominal tip radius of 2 nm. Particles solutions were diluted in a concentration of approximately 10^9 to 10^{10} particles per cm^3. Solution was dropped in fresh cleaved mica and rest to dry in vacuum chamber protected from contamination. The experiment was performed with scan resolution of 512 × 512 lines and frequency of 0.5 Hz. For indentation experiments, cantilevers of 0.57 N/m were used and maps of nano mechanical properties (Young's modulus, adhesion, energy dissipation and deformation) of particles. These maps were acquired simultaneously with particles topography using PeakForce QNM (Bruker, Santa Barbara, CA, USA) (https://www.sciencedirect.com/science/article/abs/pii/S0939641113003378).

Entrapment Efficacy (EE%)

The calculation of the entrapment efficiency (EE%) as the equation was performed by an in-house adaptation of the methodology described by Zhou *et al.* (https://www.hindawi.com/journals/jnm/2013/346274/). In this methodology we calculated the octreotide by an indirect UV visible analysis using a spectrophotometer (Shimadzu model UV-2550) with λ max value of 279 nm, where it is part of the initial value of the Octreotide incorporated in the nanoparticle and free Octreotide in the supernatant using the Eq. 1:

$$\text{Encapsulation efficiency (\%)} = [1-(\text{Drug in supernatant liquid/Total drug added})] \times 100. \quad (1)$$

In addition the loading capacity of the nanoparticles (weight %) was calculated by the Eq. 2:

$$\text{weight \%} = \text{weight of octeotride divided by the total weight of PVA/PLA.} \quad (2)$$

Cytotoxicity

Tumor Cells Cultures

Briefly, cells from human pancreas carcinoma (MIA Paca-2) that express SSRT 2 (somatostatin receptor subtype 2) and can mimicking the PPoma behavior (20,21), were obtained from Cell Bank of Rio de Janeiro (Rio de Janeiro, Brazil). The cells were routinely maintained in DMEM supplemented with 10% FBS, $NaHCO_3$ (3.7 g/L), HEPES (5.2 g/L), penicillin (0.5 U/mL) and streptomycin (0.5 mg/mL). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. Cells were grown to confluence culture flasks. Cells were detached by brief treatment with trypsin (0.1%) / EDTA (0.01%) (22).

MTT Assay

In order to evaluate if the PLA/PVA/Octreotide nanoparticles could interfere with cell survival, the MTT assay was performed. In this direction, MIA Paca-2, at concentration of 5×10^3 cells were plated on 96 wells plate and incubated with 10% FBS and incubated with 1 μM, 5 μM, 10 μM,20 μM, 50 μM and 100 μM of PLA/PVA/Octreotide nanoparticles (separately) for periods of 24, 48 and 72 h at 37°C in a humidified atmosphere with 5% CO2. After the incubation period, the culture medium was removed and cells were incubated with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution at 1 mg / ml for 2 h. Thereafter, the MTT solution was removed and 200 μL of absolute isopropanol was added to lyse the cells and solubilize the MTT crystals in all conditions. The absorbance of the extract was read at 570 nm in a reader ELISA plates. The graphs represent the mean ± standard deviation (SD) from three independent experiments. UT: Untreated cells. Statistical significance was analyzed by the one-way ANOVA test (*$p < 0.05$; **$p < 0.01$) (23).

Labeling Process with Technetium-99 m (^{99m}Tc)

The labeling process was done by the direct radiolabeling process described by Portilho *et al.* (24) and Sousa-Batista *et al.* (25). In this methodology we used 150 μL of each sample: i) loaded nanoparticle with Octreotide (PLA/PVA/Octreotide nanoparticles) and ii) unloaded (empty)nanoparticle, incubated with 150 μL of stannous chloride ($SnCl_2$) solutions (30 μg/mL) (Sigma-Aldrich) for 10 min at room temperature. Then this solution was incubated with 100 μCi (3.7 MBq) (approximately 300 μL) of ^{99m}Tc (IPEN/CNEN) for 10 min in order to label their structures, as schematically demonstrated in Fig. 1.

Quality Control of the Labeling Process with ^{99m}Tc

In order to confirm the efficacy of the nanoparticles labeling process, paper chromatography was done using Whatman paper n° 1 using 2 μL of sample I – loaded nanoparticle with Ocreteotide (PLA/PVA/Octreotide nanoparticles); II – unloaded (empty)nanoparticle and acetone (Sigma-Aldrich) as mobile phase. The radioactivity of the strips was verified in a γ-counter (Perkin Elmer Wizard® 2470, Shelton, CT City, State) (https://www.sciencedirect.com/science/article/pii/S0168365918302608).

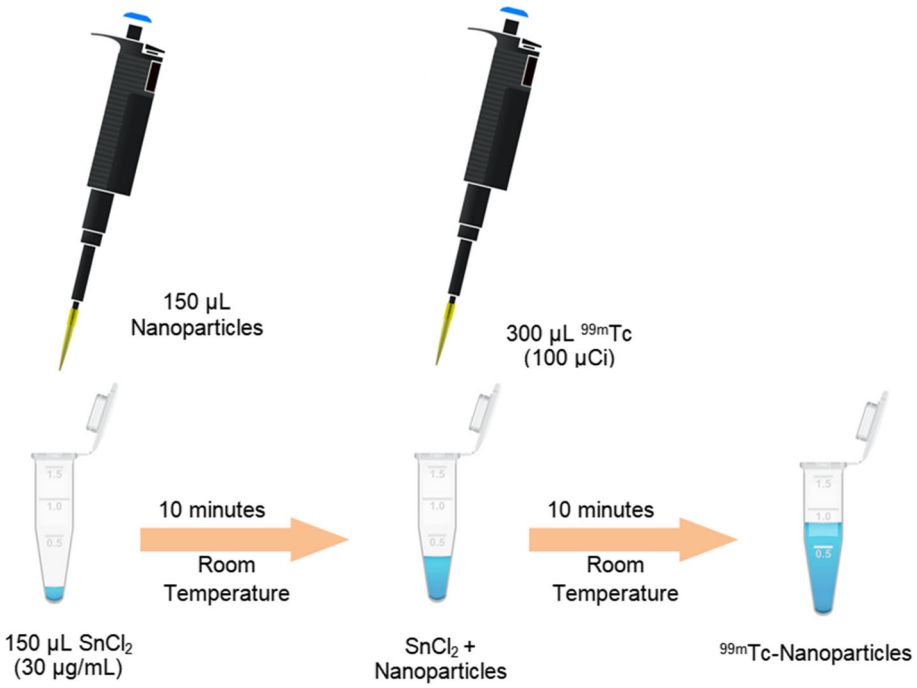

Fig. 1 Schematic labeling process of nanoparticles with 99 m-Technetium by the direct method.

Labeling Stability Test

To confirm the stability of the nanoparticles labeling process, paper chromatography was done using Whatman paper n° 1, 2 µL of sample I – loaded nanoparticle with Ocreteotide (PLA/PVA/Octreotide nanoparticles); II – unloaded (empty) nanoparticle and acetone (Sigma-Aldrich) as mobile phase in the following period of time: 0, 30′,1 h, 2 h, 4 h, 6 h and 24 h. The radioactivity of the strips was verified in a γ-counter (Perkin Elmer Wizard® 2470, Shelton, CT City, State) (26–28).

In vivo Analysis

Tumor Xenografted Models – PPoma Animal Model

Human pancreas carcinoma (MIA Paca-2) (Cell Bank of Rio de Janeiro, Brazil) were cultured in RPMI (Gibco, Life technologies, MD, USA) supplemented with 10% of fetal bovine serum (Gibco, Life technologies, MD, USA) and 50 µg/mL of gentamicin (Gibco, Life technologies, MD, USA). Mycoplasma contamination in cultured cells was excluded using Lonza Mycoplasma Detection Kit.

It ss important to state that we used the MIA Paca-2 cell line since this cell line express large amounts of SSRT 2 (somatostatin receptor type 2), which is the most prominent somatostatin receptor presented in the PPoma, once the PP (pancreatic polypeptide) cannot be used as original biomarker because his expression is marginally recognized (2,11,29–31).

Tumors were established by subcutaneous (SC) injection of 1×10^6 MIA Paca-2 cells at the right flank of eight-week-old male Balb/c nude mice ($n = 6$). Tumor size was monitored for 3 weeks and measured by a caliper. Mice were observed three times per week for evidence of distress, ascites, paralysis or excessive weight loss.

Biodistribution

For the biodistribution studies, 3.7 MBq/0.01 mL of each sample: sample I – loaded nanoparticle with Ocreteotide (PLA/PVA/Octreotide nanoparticles) and II – unloaded(empty PLA/PVA nanoparticle) nanoparticle were injected by retro-orbital via as described by De Jesus Felismino *et al.* (32) and Salvi *et al.* (33). Briefly, male Balb/c nude mice ($n = 6$ per group) were anesthetized intramuscularly with a solution mix of 10% Ketamine and 2% Xylazine and then, ^{99m}Tc-PLA/PVA/Octreotide nanoparticles as PLA/PVA nanoparticle were administered by retro-orbital injection. Two hours after administration male mice were sacrificed by asphyxiation (CO_2 chamber) and organs of interest (heart, brain, stomach, intestine, kidney, lung, liver, spleen, lesion (tumor) and blood were immediately dissected out and weighed for quantitative estimation of gamma counts using a gamma counter (Perkin Elmer, Wizard® 2470). Results were expressed as percentage of injected dose per organ (%ID/organ).

Statistical Analysis

Statistical analyses were performed using Origin Pro 8 (OriginLab, USA) software. Results are shown as means ± standard deviation (SD). *P*-values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

Nanoparticle Fabrication

The double emulsion followed by solvent evaporation method showed to be an efficient methodology for the encapsulation of hydrophilic molecules and proteins such as Octreotide (34,35). The results from DLS and AFM corroborated this efficacy, as shown below.

Dynamic Light Scattering

The PLA/PVA/Octreotide nanoparticle showed a mean size of 189 ± 1 nm with a polydispersity index (PDI) of 0.097 ± 0,022 (Fig. 2), corroborating the monodispersive behavior (36–38).

The acquired size (189 nm) is suitable for a higher EPR effect (Enhanced Permeability and Retention) in tumor cells, specifically in solid tumors, as PPoma, since with this size the PLA/PVA/Octreotide nanoparticle can reach the broader fenestrations of the neo-vessels, but cannot enter the narrow endothelial fenestrations of normal tissues (36,39–43). In this direction is expected a higher accumulation (uptake) in the tumor with low accumulation (uptake) in normal tissues (44).

Atomic Force Microscopy (AFM)

In topographical AFM images (Fig. 3), PLA/PVA/Octreotide nanoparticles showed high homogeneity in shape (spherical) and a narrow size distribution in the middle area of 180 to 200 nm, corroborating the DLS analysis.

Entrapment Efficacy (EE%)

The result from EE% was also consistent and showed that 60% of the Octreotide were incorporated into the nanoparticles, corroborating the double-emulsion methodology as a good technique to produce high-loaded nanoparticles of Octreotide. This data is corroborated by the loading capacity, which was 0,03 wt%.

Cytotoxicity

The results from the MTT assay are expressed in Fig. 4. The results corroborate the safety aspect of the nanoparticle. Also, confirmed that the amount of Octreotide used was not enough to kill the cells (MIA Paca-2). This is quite desirable especially when developing contrast/imaging agent as is the case of this nanosystem (26,27).

The results corroborate the safety and harmless of these nanoparticles and it use as imaging agent (24).

Labeling Process

The PLA/PVA/Octreotide nanoparticles were successfully labeled (> 90%). The use of acetone as the mobile phase provided efficient separation from free ^{99m}Tc and labeled nanoparticles, as shown in Table I.

This result is in agreement with the literature, which says that the amount of sample must be sufficient to bind at least 90% of the ^{99m}Tc, thus obtaining an image with few artifacts (45–47). Similar results were found for empty nanoparticles as shown in Table II.

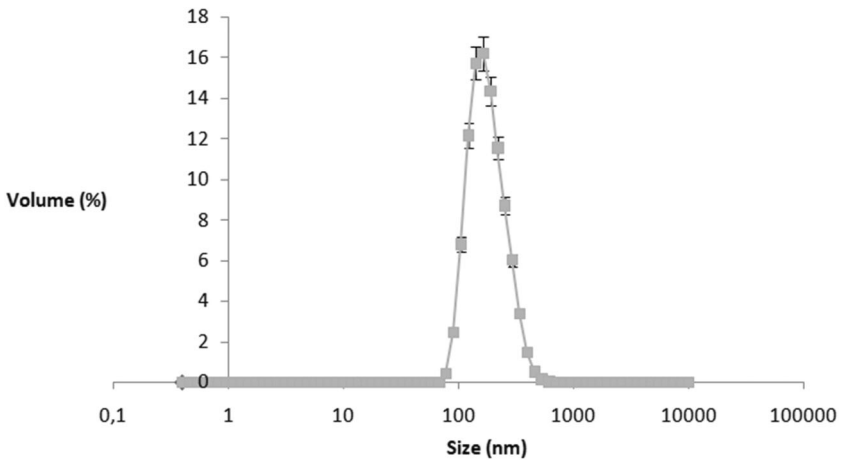

Fig. 2 PLA/PVA/Octreotide nanoparticle size distribution and mean size. Analyses were performed at 25°C after preparation. Error bars indicate SD for the triplicates.

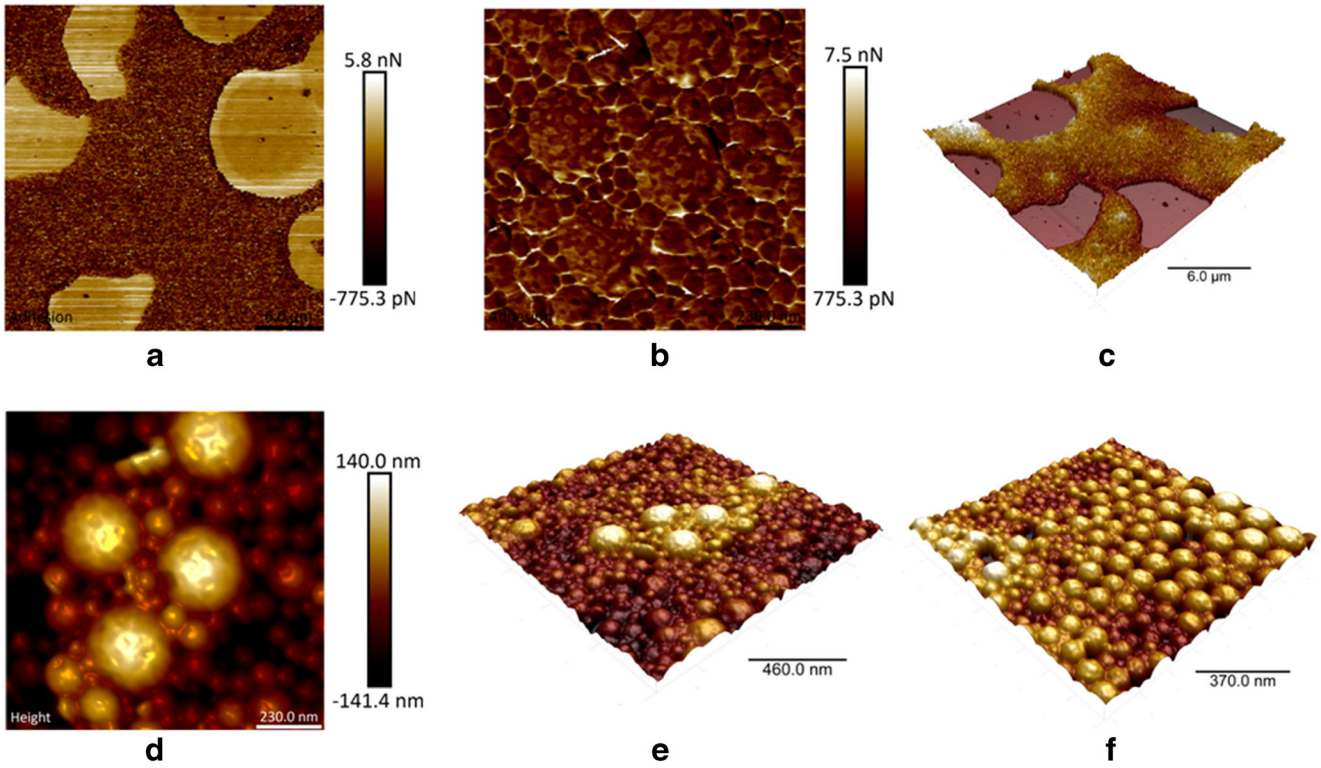

Fig. 3 Topographic image of nanoparticles obtained by atomic force microscopy showing their size and the spherical shape. (**a**) Film adhesion; (**b**) Adhesion; (**c**) Film 3D; (**d**) Height; (**e**) and (**f**) Uniformity of structures.

Biodistribution

The biodistribution (Fig. 5) of both nanosystems (^{99m}Tc-PLA/PVA/Octretide and 99mTc-PLA/PVA, respectively loaded and empty nanoparticles/nanosystems) is quite different in inducted animals and changed the behavior of the loaded nanoparticles when compared with empty nanoparticles (Fig. 5). The uptake by the liver (64.51% and 61.86%, respectively empty and load nanoparticle) was very similar and may be explained by the fact that nanoparticles are recognized by the mononuclear phagocytic system (MPS) (48–51). This theory is corroborated by the high uptake by the spleen (10.79% and 7.8%, respectively loaded and empty nanoparticles) in both cases.

A high uptake in stomach was observed in the intervention group (treated with ^{99m}Tc-PLA/PVA/Octreotide nanoparticles), which can be explained by the fact that stomach express somatostatin receptor (21). The uptake in large and small intestine in the same group was also observed and can be explained due to the high amount of SSRT 2 receptor in intestines too (21,52). In both cases a negligible uptake was observed in brain (0.06% and 0.05%, respectively empty

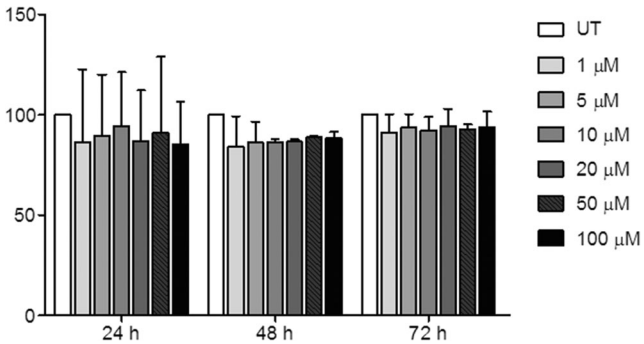

Fig. 4 MTT assay using PLA/PVA/ Octreotide nanoparticles at concentration of 1, 5, 10, 20, 50 and 100 μM, at the times of 24, 48 and 72 h. The MTT assay was performed and optical density was obtained at 570 nm. The graphs represent the mean ± standard deviation from three independent experiments. UT: Untreated cells. Statistical significance was analyzed by the one-way ANOVA test (*$p < 0.05$; **$p < 0.01$).

Table I Percentage of Labeled PLA/PVA/ Octreotide Nanoparticles Observed Over Time, After Ascending Chromatograms of ^{99m}Tc Compared with Free Pertechnetate ($Na^{99m}TcO_4$)

Time (h)	Labeling (%)
0	99.8 ± 0.2
0.5	99.7 ± 0.3
1	98.8 ± 0.6
2	99.7 ± 0.5
4	98.6 ± 0.9
6	98.7 ± 0.6
24	98.3 ± 0.8

Table II Percentage of Labeled Empty Nanoparticles Observed Over Time, After Ascending Chromatograms of ^{99m}Tc Compared with Free Pertechnetate ($Na^{99m}TcO_4$)

Time (h)	Labeling (%)
0	99.9 ± 0.4
0.5	99.8 ± 0.2
1	99.8 ± 0.6
2	99.7 ± 0.4
4	99.6 ± 0.7
6	98.2 ± 0.5
24	98.0 ± 0.2

and loaded nanoparticles) corroborating the safety aspect in terms of radioactive imaging agent.

In both groups renal uptake was observed (∑ = 14.7% and ∑ = 6.8%, respectively empty and loaded nanoparticles), but with imperceptible amount in bladder (0.9% and 1%) respectively empty and loaded nanoparticles, which suggest that the both nanosystems were re-absorbed in the luminal space instead of been excreted (53).

The uptake by the lungs (∑ = 4.5% and ∑ = 5.1%, respectively empty and loaded nanoparticles) can be explained by the administration route (33,54). Finally, the uptake in lesion (tumor) by the loaded nanosystem (5.3%) in contrast with the empty nanosystem (0.01%) corroborated the targeting of the nanosystem and its use as a nano-imaging agent for PPoma.

CONCLUSION

Although PPoma is being considered a rare type o tumor, the aggressiveness as the very low rate of treatment's success makes this cancer a challenge. Most of the issues related to PPoma rely in the difficult to early detect, since no large metabolic or physiological changes are observed in the first stages of the tumor. In this direction, the developed ^{99m}Tc-PLA/PVA/Octreotide nanoagent showed to be a precise and efficient agent capable to correctly targeting and identify this tumor. Further studies are necessary, especially in orthotopic cancer model, in which the biology of the cancer is more preserved. Also, imaging studies using Micro PET/SPECT are required to undoubtedly proof the concept.

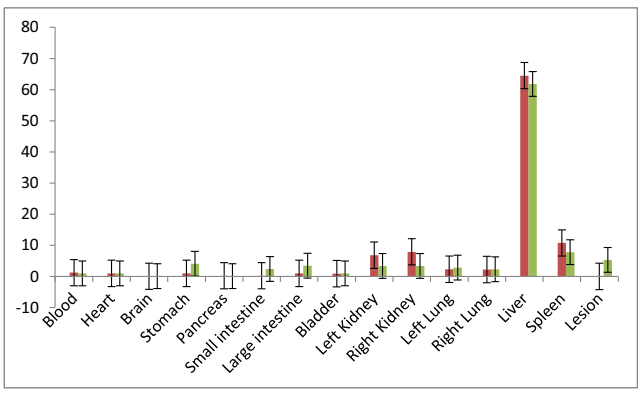

Fig. 5 Biodistribution of the ^{99m}Tc-PLA/PVA/Octreotide nanoparticles compared with the biodistribution of the ^{99m}Tc-PLA/PVA nanoparticles both with similar size in xenografted mice (n = 12, 6 animal per group) using MIA Paca-2 cell to mimic the PPoma microenvironment, with expression of somatostatin subtype 2 receptor (SSRT 2). The red group is control group (empty nanoparticles/^{99m}Tc-PLA/PVA nanoparticles) and the green group is the intervention group (loaded nanoparticles/^{99m}Tc-PLA/PVA/Octreotide).

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