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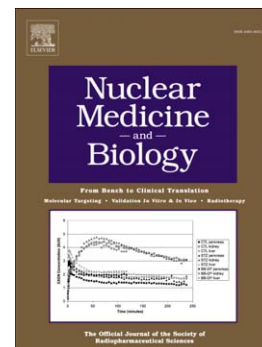
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## Radiolabeling and Biological Characterization of TPGS-based Nanomicelles by Means of Small Animal Imaging

Radiolabelled TPGS-based nanomicelles Imaging

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### Abstract

**Introduction.** In recent years, nanomedicines have raised as a powerful tool to improve prevention, diagnosis and treatment of different pathologies. Among the most well investigated biomaterials, D- $\alpha$ -tocopheryl polyethylene glycol succinate (also known as TPGS) has been on the spot for the last decade. We therefore designed a method to biologically characterize TPGS-based nanomicelles by labeling them with <sup>99m</sup>Tc. **Methods.** Labeling process was performed by a direct method. The average hydrodynamic diameter of TPGS nanomicelles was measured by dynamic light scattering and radiochemical purity was assessed by thin layer chromatography. Imaging: a dynamic study was performed during the first hour post radioactive micelles administration in a gamma camera ( $\text{TcO}_4^-$  was also administered for comparative purposes). Then two static images were acquired in ventral position: 1 hour and 12 hours post injection. Blood pharmacokinetics of <sup>99m</sup>Tc-TPGS during 24 h was performed. **Results.** Images revealed whole body biodistribution at a early and delayed time and semiquantification was performed in organs of interest (%Total counts: soft tissue

6.1±0.5; 3.9±0.1, Bone 1.2±0.2; 1±0.1, Heart 1.5±0.6; 0.7±0.3, Kidneys 16.6±1.3; 26.5±1.7, Liver 8.6±1.1; 11.1±0.1 for 1 and 12 hs post injection respectively).

**Conclusion.** This work demonstrated that TPGS based nanomicelles are susceptible to be radiolabeled with  $^{99m}\text{Tc}$  thus they can be used to perform imaging studies in animal models. Moreover radiolabeling of these delivery nano systems reveals their possibility to be used as diagnostic agents in the near future.

ACCEPTED MANUSCRIPT

## Introduction

In recent years, nanomedicines have raised as a powerful tool to improve prevention, diagnosis and treatment of different pathologies. Their nano-sized range along with their high surface-to-volume ratio and their favorable physico-chemical characteristics makes them suitable for modulation of the pharmacokinetic and pharmacodynamic drug profiles. In this context nanotechnology offers a variety of strategies to develop novel drug delivery systems with not only an improved drug aqueous solubility and chemical stability, but also with controlled drug release and preferential accumulation in certain tissues or in solid tumors (passive or active targeting) [1, 2].

Among the most well investigated biomaterials, D- $\alpha$ -tocopheryl polyethylene glycol succinate (also known as TPGS) has been on the spot for the last decade. It is the water-soluble form of vitamin E, resulting from the esterification of vitamin E succinate with polyethylene glycol 1000. It exhibits an average molecular weight of 1,513 Da and a hydrophile-lipophile balance value of 13.2 (amphiphilic nature). TPGS is a Generally Regarded As Safe (GRAS)-listed oral supplement which has been approved by the FDA as a safe pharmaceutical adjuvant used in drug formulation [3].

Due to TPGS properties as an absorption/permeation enhancer, emulsifier, solubilizer and stabilizer agent, it has been investigated for the development of a wide variety of drug delivery systems including micelles, TPGS-based nanoparticles (NPs), liposomes, nanosuspensions, solid dispersions and TPGS-based pro-drugs. For instance, TPGS-based block copolymers NPs have been studied to antineoplastic drugs delivery [4]. Also TPGS surface-decorated NPs

have been employed for paclitaxel delivery [5]. Due to TPGS amphiphilic nature, it has been explored as a micelle-forming biomaterial, especially for oral and parenteral administration in antineoplastic therapy [6-10]. In this context, it has been reported that TPGS could serve as a pharmaceutical additive for overcoming multidrug resistance and as an inhibitor of P-glycoprotein for increasing the cytotoxicity and bioavailability of anticancer drugs [5, 11, 12]. It has also been investigated for the treatment of other pathologies including the drug delivery of antiretrovirals and calcium channel blockers [13, 14].

Other properties explored for TPGS are: i) its ability to act as an adjuvant in vaccine systems for intranasal administration [15] and ii) its use as a nutrition supplement [16].

Recently, a new generation of multifunctional nanocarriers for simultaneous diagnosis and therapy, known as “theranostics” nanoformulations, has risen representing a new platform for personalized nanomedicines. These nanoformulations allow the assessment of drugs biodistribution and accumulation in a certain target along with the possibility of reaching therapeutic efficacy [17, 18]. Labeling TPGS or any of the TPGS-based nanostructures stands as a real challenge and the first rational step towards building a true theranostic agent. This first step is evaluated in this work and further research is needed to test loading (with a therapeutic drug) and labeling of TPGS-based nanostructures altogether. In addition, small animal imaging represents a powerful tool to follow up labeled probes and could help with characterization of nano-systems as it can show in vivo kinetics and organ biodistribution [19, 20]. These techniques can also provide information about in vivo stability of labeled compounds or structures. We therefore

designed a method to biologically characterize TPGS-based nanomicelles by labeling them with  $^{99m}\text{Tc}$ , a widely known and used radionuclide with diagnostic purposes in Nuclear Medicine [21]. Radiolabeling TPGS-based nanomicelles has an enormous potential for multiple purposes: first it can easily enlighten the pharmacokinetic and pharmacodynamic of this nanosystem by using noninvasive techniques, second it could also constitute a tumor diagnostic agent for Nuclear Medicine and finally and even more significant it could, potentially and with further research needed, constitute itself a theranostic agent if it is susceptible to carry a therapeutic drug and to stay labeled.

## Materials and Methods

### *Micelles preparation*

For the TPGS nanomicelles preparation, 10 g of commercial TPGS was weighted and dissolved in 100 mL of distilled water with continuous agitation and temperature (30°C) until a homogeneous dispersion was achieved. Then samples were equilibrated for 24 h and filtered by clarifying filters (0.2  $\mu\text{m}$ , cellulose nitrate) before use. Finally, the suspension was used to perform the radiolabeling procedure.

### *Micellar size distribution and morphology*

The average hydrodynamic diameter ( $D_h$ ) of TPGS nanomicelles (10% w/v) was measured by dynamic light scattering (DLS, scattering angle of  $\theta = 173^\circ$  to the incident beam, Zetasizer Nano-Zs, Malvern Instruments, United Kingdom) at 25 °C. Samples equilibrated for 5 min at 25 °C before the measurements ( $n=3 \pm \text{S.D.}$ ).

The morphology of the TPGS nanomicelles (10% w/v) was characterized by transmission electron microscopy (TEM, Philips CM-12 TEM apparatus, FEI Company, The Netherlands). Briefly, aliquots were placed onto a grid and covered with Formvar film, stained with 5  $\mu$ L of phosphotungstic acid solution (1% w/v), washed with distilled water (5  $\mu$ L) and dried into a silicagel container.

#### *Radiolabeling*

Labeling process was performed by a direct method as follows: a 10% w/v TPGS dispersion was incubated for 2 h with 25 mCi of  $^{99m}\text{TcO}_4^-$  eluted from a  $^{99}\text{Mo}$ - $^{99m}\text{Tc}$  generator (Laboratorios BACON, Argentina).  $\text{SnCl}_2$  (Sigma-Aldrich, USA) was dissolved using HCl 6 N (Anedra, Argentina) and then added to the reaction vial reaching a concentration of 2.5 mg/mL. The radiolabeling was assessed prior to  $^{99m}\text{Tc}$ -TPGS based nanomicelles intravenous administration by using paper chromatography: Whatman N°1 (W&R BALSTON Ltd, England) paper and methyl-ethyl-cetone (Cicarelli, Argentina) as stationary and mobile phases respectively.

#### *Serum Stability*

Serum samples (3 h post injection) were assessed by paper chromatography: Whatman N°1 (W&R BALSTON Ltd, England) paper and methyl-ethyl-cetone (Cicarelli, Argentina) as stationary and mobile phases respectively.

#### *Animals*

All animal procedures were approved by the Ethical Committee for the Use and Care Laboratory Animals (CICUAL) of the School of Pharmacy and Biochemistry, Universidad de Buenos Aires (Res CD 3761/13).

Sprague Dawley rats (250-300 g) were kept in stainless steel cages under a 12 h light 12 h darkness cycle. Food and water were available ad libitum. Animals were anesthetized using ketamine-xilazine (90 mg/Kg-5 mg/Kg) [22] and placed ventrally over the collimator for the administration of 74 MBq of radioactive micelles (N=3) or free radiolabel (N=3) through the tail vein (intravenously). Pressure was applied until hemostasis was achieved. After dynamic imaging and 1 h static acquisition, animals were kept in the same conditions but in a specially adapted bunker until the 12 h image.

#### *Imaging and Pharmacokinetics*

Small animal imaging was performed using a small field of view gamma camera (OHIONUCLEAR, Software; IM512P, ALFANUCLEAR, Argentina) equipped with a high resolution parallel hole collimator. A dynamic study was performed during the first hour post radioactive micelles administration. Then two static images were acquired in ventral position: 1 hour and 12 h post injection. Dynamic study: 1 frame/second (for 60 seconds) and then 1 frame/3 min (60 minutes); 256x256 matrix, 1.5 zoom. Static studies: 256x256 matrix; 1.5 zoom; more than 1.5 million counts (20-35 min scan). To analyze the results regions of interest (ROIs) were created on relevant organs in order to get semi quantitative results of the radioactive micelles biodistribution and they were expressed as % Total counts (calculated as:  $ROI_{counts}/Total_{counts} \times 100$ ).



In order to correlate the imaging results with pharmacokinetics of  $^{99m}\text{Tc}$ -TPGS based nanomicelles serum samples were taken (100  $\mu\text{L}$ ) at different time points post injection (10 min, 40 min, 1 h, 2 h, 3 h, 6 h, 12 h and 24 h) and radioactivity concentration was measured in a solid scintillation counter. Appropriate standards were built in order to express the results as %Injected dose.

## Results

The spherical morphology and size of TPGS-based nanomicelles is shown in Figure 1. The difference on micellar size between both techniques could be attributed to the micellar concentration used in this study. For the microscopy assay samples were dried, therefore, micellar aggregates could be expected since micelles are considered “dynamic systems” where their dilution can modify their hydrodynamic diameter and morphology [23, 24]. Paper chromatography results revealed  $95\pm 5\%$  of the radioactivity at the strip origin, showing the success of the radiolabeling method given that the predictable impurities remain below the commonly accepted limits for this kind of probes [25]. The results of the *in vivo* stability in serum samples ( $98\pm 1\%$ ) were in harmony with some of the imaging results which show that radiochemical purity assay was suitable for this purpose.

Figure 2 shows the *in vivo* localization of TPGS-based nanomicelles at 1 hour post administration. Nanomicelles essentially reached heart, liver, kidneys, bone and bladder by the first hour of biodistribution. This constitutes evidence of the accumulation of the micelles in organs which have cells from reticuloendothelial system (bone marrow and liver) but it also shows a possible elimination of the renal pathway and a time dependent localization in cardiac tissue. Figure 3 shows TPGS

biodistribution after 12 hours of administration, kidneys and liver increased their TPGS nanomicelles content whereas heart decreased it. A small abdominal uptake can be noticed probably due to intestinal content. Further metabolic studies should be performed in order to characterize elimination pathways and their kinetics in between the time points evaluated here and even longer ones.

Intravenous administration is a key factor when analyzing pharmacokinetics of a new nano-system. In this case, TPGS based nanomicelles were radiolabeled with  $^{99m}\text{Tc}$  which allowed follow up of the biodistribution of the  $^{99m}\text{Tc}$ -TPGS since its administration. Figure 4 and 5 show the first seconds of the intravenous administration of radiolabelled nanomicelles and the free radiolabel ( $^{99m}\text{TcO}_4^-$ ) as a control. It is noticeable the moment in which the radiopharmaceutical and the free label reach the heart (few seconds post injection) and from this moment on they began to distribute throughout the whole body. This is the perfusion phase which lasted for 1 minute (only the first seconds are shown).

Figure 6 and 7 show functional phases of  $^{99m}\text{Tc}$ -TPGS and  $^{99m}\text{TcO}_4^-$  respectively. The upper panel (from 1.5 to 7 min) shows early biodistribution and the lower, the later one (from 47.5 to 53 min). There are clear and very noticeable differences among  $^{99m}\text{Tc}$ -TPGS and the free label in their biodistribution and pharmacokinetics at the early and the latter stages. It is worth mentioning the high  $^{99m}\text{TcO}_4^-$  uptake in stomach and mouth. However, these two sites do not appear in  $^{99m}\text{Tc}$ -TPGS images, proving the labelling of the nanomicelles and the absence of free radiolabel. As mentioned above, those results were predicted accurately with radiochemical purity assay. On the light of that result it can be concluded that the labeling of the TPGS nanomicelles remains stable *in vivo* (as it was also

demonstrated by serum stability assay), at least until specific tissues were reached. Table 1 shows semi quantitative analysis of the organs of interest. At 12 hours post injection the activity accumulated in kidneys and liver was about 40% higher than at the first hour meaning that micelles continued accumulating in both organs in a time dependent way. Bone uptake was evaluated by placing the regions of interest (ROIs) over the knees which rendered similar values at both time points. An interesting fact is that soft tissue as well as cardiac tissue had almost 50% less activity at the delayed image when compared to the early one. This could reflect that at the first hour micelles were still in circulation and 12 hours later most of them were already localized in specific tissues. An extended circulating time is expected for these PEG exposing nanomicelles [26]. Moreover a two phase decay curve was fitted in  $^{99m}\text{Tc}$ -TPGS serum concentration over time as it can be seen in Figure 8. This means that clearance from blood has different constant rates during early time points when compared to delayed time points after administration. This shows that static images taken at 1 and 12 h were representative of both phases.

## Discussion

It is worth mentioning the fact that placing ROIs in planar images can lead to structure superposition which is an inherent characteristic of the semiquantification performed using this technique. Some regions are more critical than others, nevertheless biological distribution results can be obtained with all the advantages of a non invasive imaging practice.

The application of this imaging technique allows performing the biological characterization of TPGS nanomicelles, being these data useful to predict their application on different pathologies, as a potential diagnostic tool and a drug delivery system for passive or active targeting in infectious reservoirs (theranostic agent). Among the different infectious diseases which present anatomical reservoirs, extrapulmonary tuberculosis (epTB) (affecting 50% of human immunodeficiency virus (HIV)-positive patients) exhibits a skeletal/articular form (35% of epTB cases) which involves arthritis and extraspinal osteomyelitis. Also it has been described renal tuberculosis, being the kidneys the most affected organs and tuberculous pericarditis [27, 28]. In case of HIV antiretroviral therapy, the overcoming of latent HIV reservoirs throughout the body involves one of the main objectives of the present Highly Active Antiretroviral Therapy (HAART). Indeed, the gut- and rectal associated lymphoid tissue as well as the intestinal macrophages have been described as viral reservoirs for HIV type 1 [29]. Among the parasite diseases, malaria caused by *Plasmodium vivax* (a worldwide severe malaria form) also is being on the spot in past years due to the description of infection relapse by liver hypnozoites activation. In this framework, liver accumulation of TPGS micelles could enhance the current therapy towards malaria elimination [30].

Thereby this radiopharmaceutical approach provides biological behavior understanding of the nanomicelles in a way no other technique does. Radionuclide based imaging contributes, in this way, to preclinical phases of new drugs or delivery systems development. The information provided and the clinical translation of this imaging technique is not comparable to any other practice. The key lays on the ability to know specific organ localization at precise time points which could

help elucidate the true potentialities of this nanosystem avoiding theoretical speculations. In addition, research in this area raises awareness of the possible side effects of its administration. Serum concentration studies allow us to understand pharmacokinetics of this radiolabelled probe and to validate the imaging time points chosen. Therefore we consider them complementary studies to imaging assessment.

This work demonstrated that TPGS based nanomicelles are susceptible to be radiolabelled with  $^{99m}\text{Tc}$ , thus they can be used to perform imaging studies in animal models. Interestingly, these novel nanosystems could be successfully employed as a potential diagnostic agent. Furthermore, commercially available FDA approved-biomaterials could offer the possibility of develop cost-viable and scalable products without the requirement of long-term/expensive biocompatibility assays. This issue stands as extremely useful for the potential bench-to-bed research translation employing TPGS as a biomaterial.

### Supporting Information

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		%Total counts					
		Soft Tissue	Bone	Heart	Kidneys	Liver	Background
<b>1 h</b>							
<b>PI</b>		6.1±0.5	1.2±0.2	1.5±0.6	16.6±1.3	8.6±1.1	0.15±0.01
<b>12 h</b>							
<b>PI</b>		3.9±0.1	1.0±0.1	0.7±0.3	26.5±1.7	11.1±0.1	0.080±0.002

**Table 1.** Semiquantification of the %Total for specific organs. Results were calculated by creating ROIs. Two different time points are presented: 1 and 12 hours post radiolabelled nanomicelles injection (PI).

**Figure 1.** Size distribution (a) and TEM micrographs (b) of TPGS nanomicelles (10% w/v), scale bar: 100 nm. (c) Macroscopic aspect of a TPGS micellar dispersion in distilled water.

**Figure 2.** Biodistribution of  $^{99m}\text{Tc}$  radiolabelled TPGS-based nanomicelles (74 MBq). Static image was acquired 1 hour post administration in ventral view (256 x 256 matrix, 1.5 zoom,  $\geq 1.5$  million counts, 20 min scan). Anesthesia: ketamine/xilazine. Color Band scale is shown.

**Figure 3.** Biodistribution of  $^{99m}\text{Tc}$  radiolabelled TPGS-based nanomicelles (74 MBq). Static image was acquired 12 hours post administration in ventral view (256 x 256 matrix, 1.5 zoom,  $\geq 1.5$  million counts, 35 min scan). Anesthesia: ketamine/xilazine. Color Band scale is shown.

**Figure 4.** Perfusion Phase of the dynamic study of  $^{99m}\text{Tc}$ -TPGS. The study began with the radiopharmaceutical intravenous administration. Acquisitions were made as 1 second /frame (256 x 256 matrix, 1.5 zoom, 60 seconds). Numbers below each frame represent the seconds post injection. Anesthesia: ketamine/xilazine. Standard scale is shown.

**Figure 5.** Perfusion Phase of the dynamic study of  $^{99m}\text{TcO}_4^-$ . The study began with the free label administration. Acquisitions were made as 1 frame/second (256 x 256 matrix, 1.5 zoom, 60 seconds). Numbers below each frame represent the seconds post injection. Anesthesia: ketamine/xilazine. Warm scale is shown.

**Figure 6.** Functional Phase of the dynamic study of  $^{99m}\text{Tc-TPGS}$  (37 MBq). The study began 1 minute post radiopharmaceutical intravenous administration. Acquisitions were made as 30 seconds/frame (256 x 256 matrix, 1.5 zoom, 60 minutes). Numbers below each frame represent the minutes post injection. Anesthesia: ketamine/xilazine. Standard scale is shown.

**Figure 7.** Functional Phase of the dynamic study of  $^{99m}\text{TcO}_4^-$  (37 MBq). The study began 1 minute post radiopharmaceutical intravenous administration. Acquisitions were made as 30 seconds/frame (256 x 256 matrix, 1.5 zoom, 60 minutes). Numbers below each frame represent the minutes post injection. Anesthesia: ketamine/xilazine. Yellow scale is shown.

**Figure 8.** Pharmacokinetics of  $^{99m}\text{Tc-TPGS}$  based nanomicelles.  $^{99m}\text{Tc-TPGS}$  was injected (350  $\mu\text{Ci}$ ) intravenously by the tail vein. Serum concentration (100  $\mu\text{L}$  sample) was expressed as a % Injected dose (%ID) and it is plotted as  $\text{media} \pm \text{SD}$  (N=3; error bars that cannot be seen are smaller than dots). A non linear curve (two phase decay) was fitted and parameters are shown: constant rates for fast and slow phases ( $K_{\text{fast}}$ ,  $K_{\text{slow}}$  respectively); extrapolated %Injected dose at 0 h (%ID<sub>0</sub>); Plateau: %ID value at theoretical infinite times (PI).

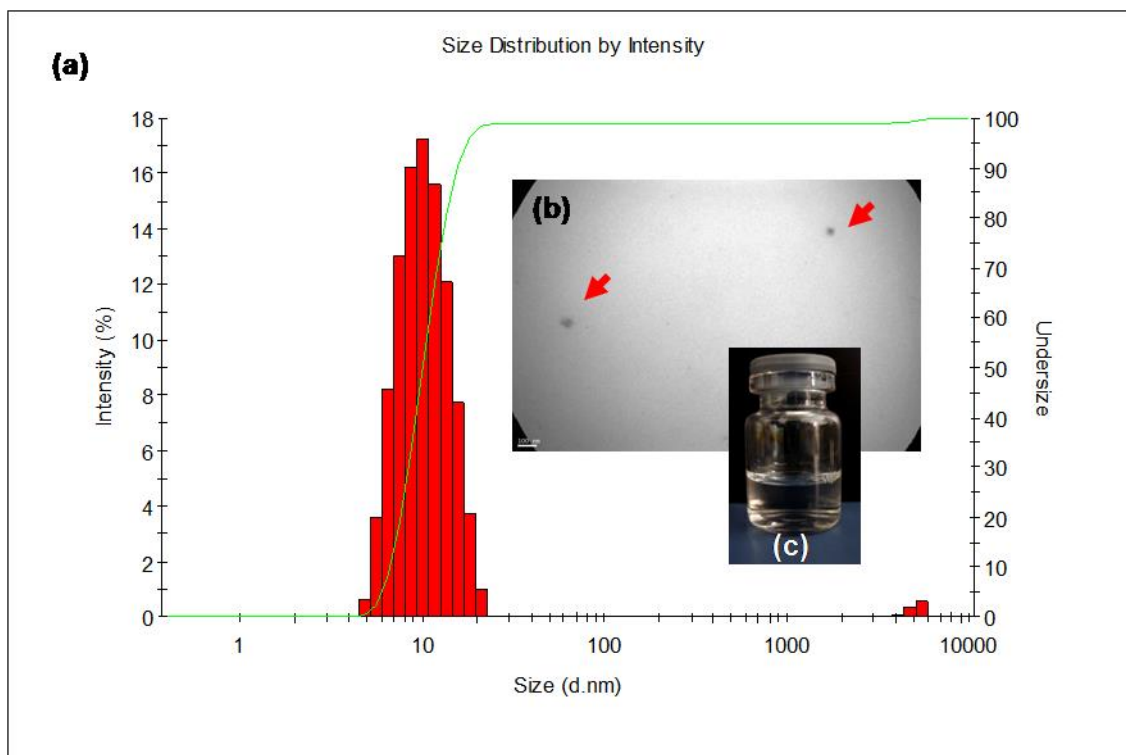


Fig. 1

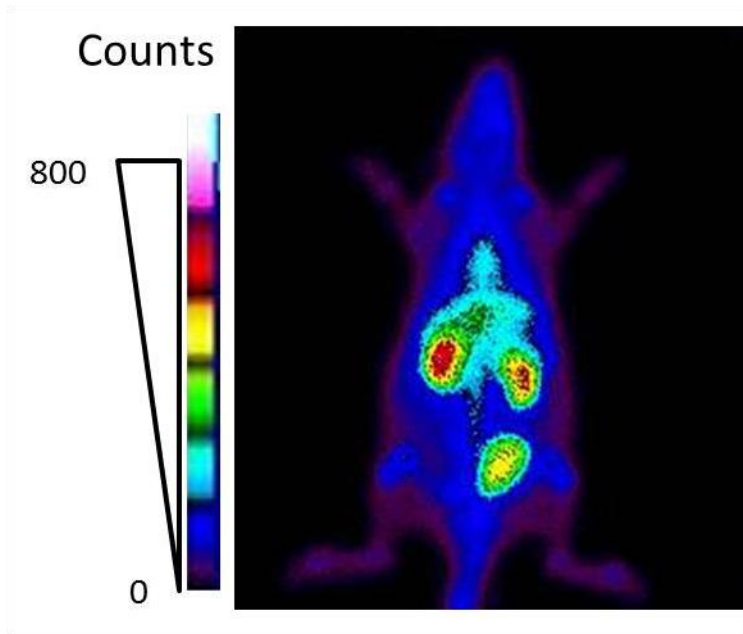


Fig. 2

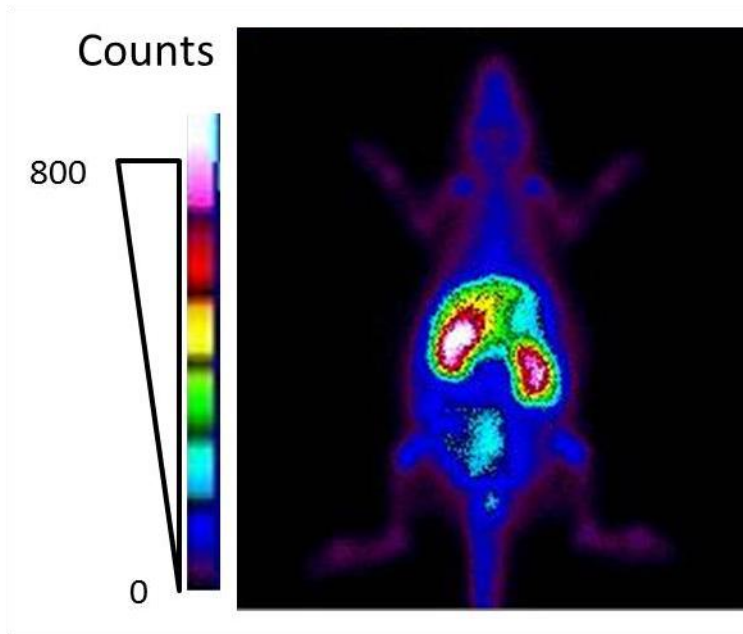


Fig. 3

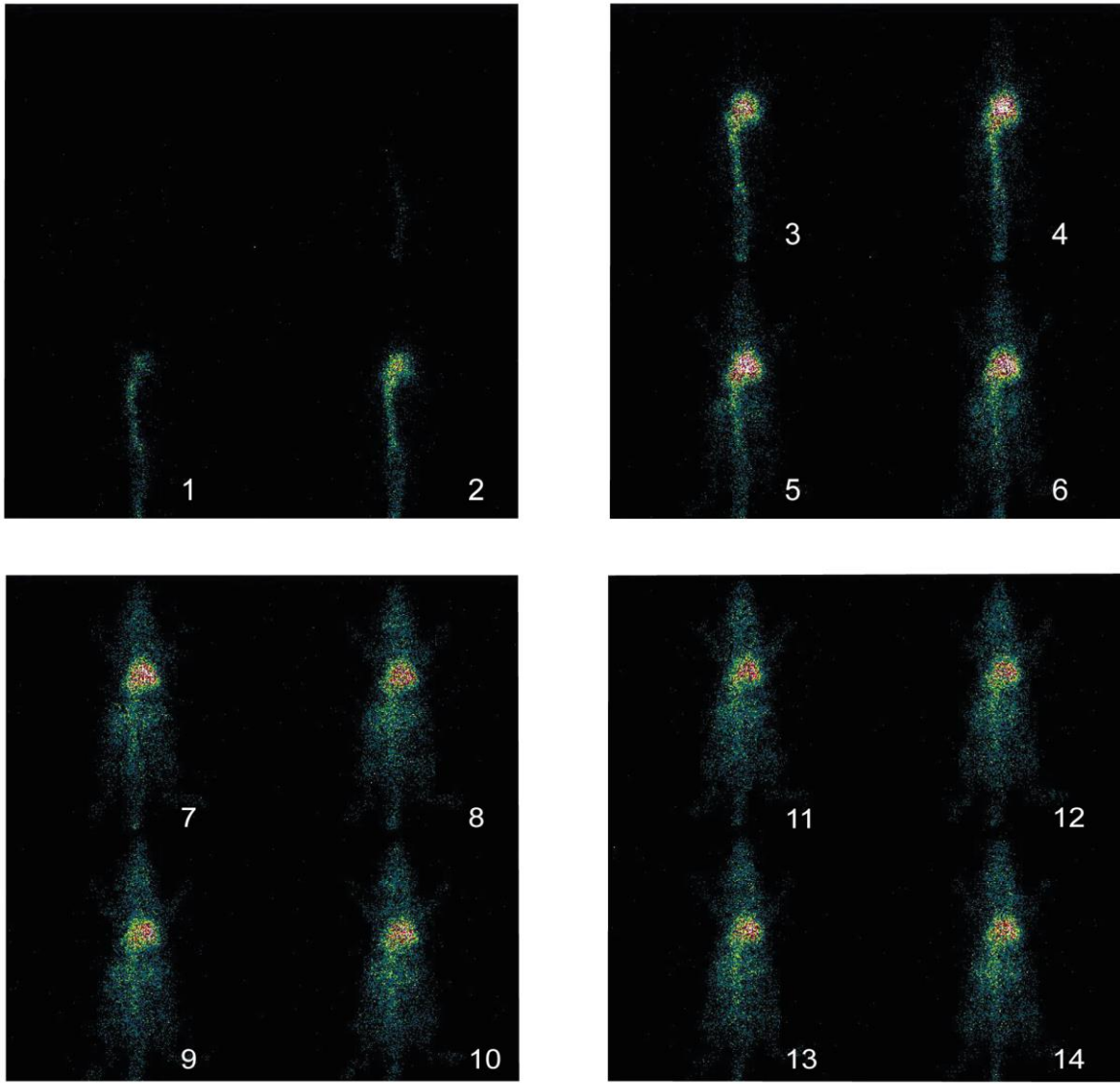


Fig. 4

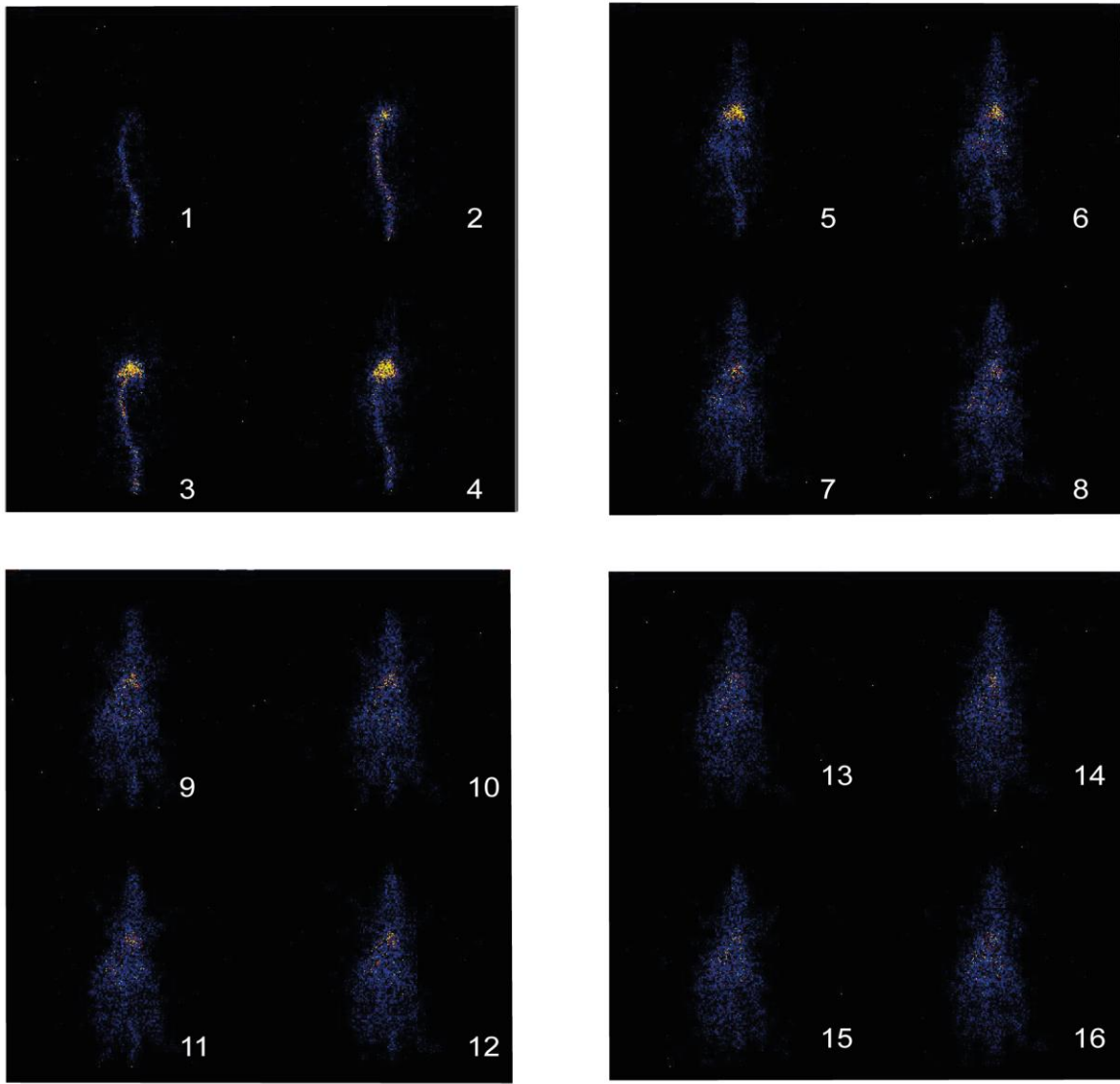


Fig. 5



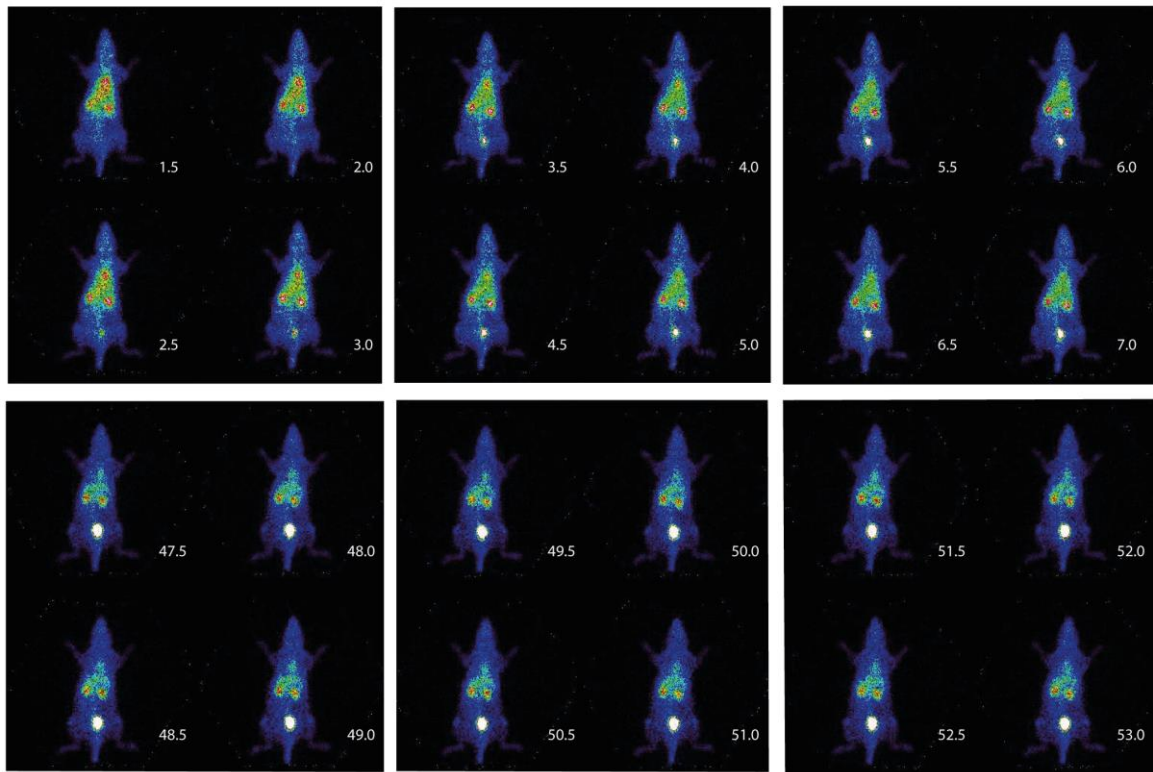


Fig. 6

ACCEPTED

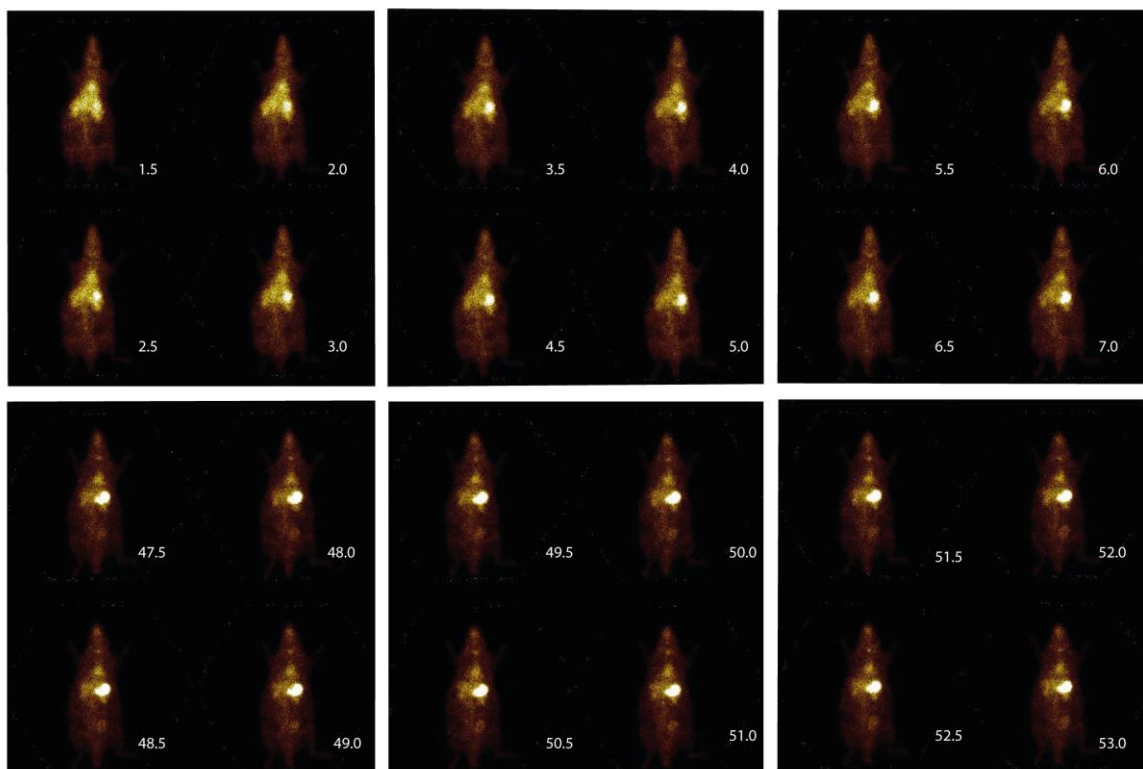


Fig. 7

ACCEPTED

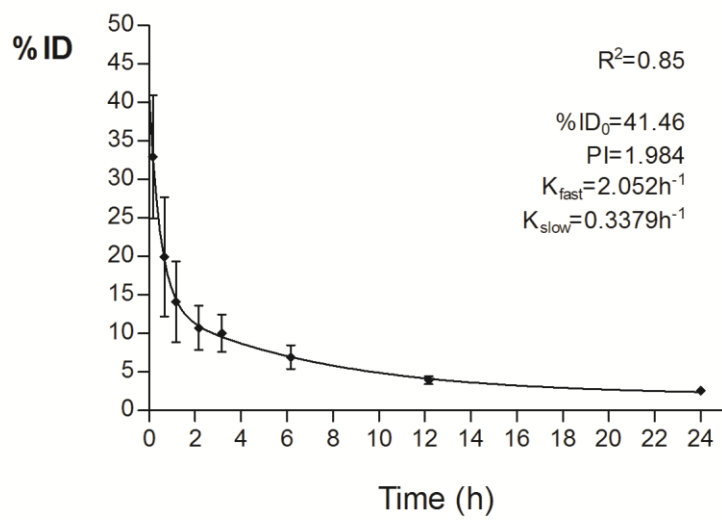


Fig. 8