Biodistribution of $^{68/67}$Ga-Radiolabeled Sphingolipid Nanoemulsions by PET and SPECT Imaging

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**Background and Purpose:** Non-invasive imaging methodologies, especially nuclear imaging techniques, have undergone an extraordinary development over the last years. Interest in the development of innovative tracers has prompted the emergence of new nanomaterials with a focus on nuclear imaging and therapeutical applications. Among others, organic nanoparticles are of the highest interest due to their translational potential related to their biocompatibility and biodegradability. Our group has developed a promising new type of biocompatible nanomaterials, sphingomyelin nanoemulsions (SNs). The aim of this study is to explore the potential of SNs for nuclear imaging applications.

**Methods:** Ready-to-label SNs were prepared by a one-step method using lipid derivative chelators and characterized in terms of their physicochemical properties. Stability was assessed under storage and after incubation with human serum. Chelator-functionalized SNs were radiolabeled with $^{67}$Ga and $^{68}$Ga, and the radiochemical yield (RCY), radiochemical purity (RCP) and radiochemical stability (RCS) were determined. Finally, the biodistribution of $^{67/68}$Ga-SNs was evaluated in vivo and ex vivo.

**Results:** Here, we describe a simple and mild one-step method for fast and efficient radiolabeling of SNs with $^{68}$Ga and $^{67}$Ga radiosotopes. In vivo experiments showed that $^{67/68}$Ga-SNs can efficiently and indistinctly be followed up by PET and SPECT. Additionally, we proved that the biodistribution of the $^{67/68}$Ga-SNs can be conveniently modulated by modifying the surface properties of different hydrophilic polymers, and therefore the formulation can be further adapted to the specific requirements of different biomedical applications.

**Conclusion:** This work supports $^{67/68}$Ga-SNs as a novel probe for nuclear imaging with tunable biodistribution and with great potential for the future development of nanotheranostics.

**Keywords:** sphingomyelin nanoemulsions, biodistribution, gallium-68/67, nuclear imaging, nanotheranostics

**Introduction**

Over the last decades, personalized medicine has greatly evolved with the development of imaging tools that improve the management of several diseases, especially cancer. Among all the non-invasive imaging techniques, the nuclear imaging modalities Single Photon Emission Computed Tomography (SPECT) and Positron Emission Tomography (PET) stand out mainly due to their high sensitivity prospect of obtaining quantitative information. In fact, PET and SPECT imaging can provide detailed information about the in vivo behavior and pharmacokinetics of several compounds, such as nanomedicines, and can facilitate their translation to clinics.
On the other hand, nanomedicine has emerged as a promising strategy to improve diagnosis and treatment of prevalent diseases including cancer.\(^3\,^4\) One of the most promising advantages of nanomedicine is the possibility to combine therapeutic molecules with diagnostic agents into single multifunctional nanoparticles, known as nanotheranostics, opening an entirely new field of development towards the implementation of personalized medicine.\(^5\,^6\) In recent years, the combination of nanoparticles with radionuclides is rapidly growing and there are a great number of submissions for the Food and Drug Administration approval.\(^7\) Different types of nanoparticles are investigated for nuclear medicine applications and multimodal imaging.\(^8\) For example, inorganic nanoparticles have been widely studied due to their intrinsic physical properties, that convert them into materials with a high potential for multimodal imaging.\(^9\,^10\) Nevertheless, organic nanoparticles are still the most in demand for the development of imaging probes by virtue of their biodegradable and biocompatible composition, preventing a long-term accumulation in the body and undesirable toxic side effects.\(^11\) Indeed, liposomes are the most extended type of organic nanoparticles for nuclear imaging applications. Liposomes can be radiolabeled by different methodologies, which can be adapted to different kinds of nanoparticles, such as micelles, solid lipid nanoparticles, and nanoemulsions.\(^12\) Chelator-based radiolabeling strategies offer a high versatility for the incorporation of radionuclides with different properties, suitable for complementary imaging techniques and nanotheranostics.\(^13\)

Nanoemulsions are defined as nanoscale droplets in which two immiscible liquids are mixed to form a single phase. Their biocompatible composition, easy production by soft and scalable methodologies and improved drug loading capacity compared to liposomes, are relevant advantages that have prompted their use in biomedicine.\(^14\,^15\) Nanoemulsions have been widely studied for fluorescence, MRI and ultrasounds imaging.\(^16\,^17\) However, their use in nuclear imaging is still recent and there are only few reports describing radiolabeled nanoemulsions.\(^18\,^19\) Our group has recently reported the development and characterization of sphingomyelin nanoemulsions that incorporate sphingomyelin, one of the main lipids in cell membranes (SNs), and claimed their potential in drug delivery.\(^20\) The principal advantages of SNs relate to their safe and simple composition, long-term colloidal stability, and capacity for accommodation of different types of functionalities and therapeutic payloads.\(^21\,^22\)

Previous attempts by our research group have proved that SNs can be radiolabeled with Fluorine-18 for PET imaging following a maleimide reaction. However, radiochemical yields (RCY) were found to be highly dependent on the crosslinking efficacy.\(^23\) The aim of this work was to provide an optimized composition and straightforward methodology for the radiolabeling of SNs with \(^{68}\)Ga and \(^{67}\)Ga radioisotopes using a chelator-based strategy. Radiolabeled formulations can be used for indistinct application in PET and SPECT imaging, and therefore adaptable to specific needs and biomedical applications.
**Materials and Methods**

**Synthesis and Characterization of NOTA-Stearylamine Derivative**

Octadecylamine (stearylamine, >99%, Merck Group, Darmstadt, Germany) was conjugated to 2-(4-isothiocyanato-5-benzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (pSCN-Bn-NOTA, NOTA, >94%, Macrocyclics, Dallas, TX, USA) to obtain a lipid derivative chelator for further inclusion into the nanoemulsions. Details about the reaction protocol and the product characterization are included in the Supporting Information.

**Preparation of Sphingomyelin Nanoemulsions**

SNs were prepared following a method previously reported by our group with minor modifications. Briefly, oleic acid (5 mg, 65–88%, Merck Group, Darmstadt, Germany), egg sphingomyelin (0.5 mg, 98%, Lipoid GmbH, Ludwigshafen, Germany) and the surfactant C16/C18-COO-C16/C18-COO-C16/C18-COO (98%, Lipoid GmbH, Ludwigshafen, Germany) were dissolved in 100 µL of absolute ethanol (99.7%, Cienytech S.L., A Coruña, Spain) with a lipid ratio 1:0.1:0.1 w/w. Then, this organic phase was injected into 1 mL of MilliQ water (Millipore Milli-Q system) under magnetic stirring using an insulin syringe (0.5 mL, 0.33 x 12 mm ICO.C.1) and nanoemulsions (SNs, DTPA-SNs, NOTA-SNs or NOTA-PEG-SNs) were spontaneously formed. To prepare SNs coated with hyaluronic acid (NOTA-HA-SNs), the organic phase containing the lipids and NOTA was injected under stirring in 1 mL of an aqueous solution of sodium hyaluronate (HA, 170 kDa, 2 mg mL⁻¹, >95%, Bioiberica, S.A.U, Barcelona, Spain).

**Physicochemical and Morphological Characterization**

All the nanoemulsions were physicochemically characterized using a Nanosizer 2000® (Malvern Instruments, Malvern, UK). The mean size and its distribution, defined by the polydispersity index (PDI), were measured by Dynamic Light Scattering (DLS). Measurements were performed on disposable microcuvettes (ZEN0040, Malvern Instruments) upon dilution of the SNs in MilliQ water, reaching a final lipid concentration of 0.5 mg mL⁻¹. The zeta potential (ZP) was analyzed by Laser Doppler Anemometry (LDA) diluting SNs in MilliQ water (lipid concentration 0.12 mg mL⁻¹) with Folded capillary cuvettes (DTS1070, Malvern Instruments). The stability of SNs, DTPA-SNs and NOTA-SNs was tested under storage conditions at 4 °C up to one month and also after incubation with human serum at 37 °C for 72 h. The colloidal properties were measured by DLS maintaining the conditions mentioned before. Parameters such as the medium (water) and the temperature (25 °C) were fixed for all the measurements.

The morphology of SNs was observed by Field Emission Scanning Electron Microscopy (FESEM) using a ZEISS FESEM ULTRA Plus, microscope (Carl Zeiss Micro Imaging, GmbH, Germany). Before the measurement, 20 µL of sphingomyelin nanoemulsions (0.5 mg mL⁻¹) were stained with 20 µL phosphotungstic acid (2% w/v). Then, 20 µL of the mixture was placed on a carbon coated grid and left for 2 minutes. The excess was removed using a filter paper and the grid was allowed to dry. The grid was washed 5 times with 100 µL of filtered MilliQ water and it was dried overnight.

**Cell Culture Conditions**

A549 (ATCC® CCL-185), MDA-MB-231 (ATCC® HTB-26) were cultured in Dulbecco’s modified Eagle's medium high glucose (DMEM, Merck Group, Darmstadt, Germany) and OMM-2.5 (kindly provided by Martine J. Jager from Leiden University Medical Center, Leiden, The Netherlands) were grown in RPMI (Gibco, Thermo Scientific S.L., Waltham, MA, United States). Cells were maintained at 37 °C with 95% relative humidity and 5% CO₂.

**Cellular Uptake of SNs**

Cellular uptake of SNs was studied by confocal microscopy. 8x10⁴ cells/well were seeded in an 8-well μ-chamber (SPL Life Sciences Co., Ltd., Gyeonggi-do, Korea). After 24 h, cells were treated with SNs (0.13 mg mL⁻¹ per well) labeled with C11-TopFluor sphingomyelin (>99%,...
Avanti Polar Lipids, Alabama, Al, USA). After 4 h of incubation at 37 °C, cells were washed with 1X phosphate buffer saline (PBS) twice and fixed with 4% paraformaldehyde for 15 min. Cells were then washed twice with 1X PBS and the cellular nuclei were counterstained with Hoechst 33,342 (Thermo Scientific S.L., Waltham, MA, United States) for 5 min. After washing, the slide was mounted with Mowiol (Merck Group, Darmstadt, Germany) and a coverslip. The samples were left to dry in the dark overnight at RT, following their storage at −20 °C, until taken for observation under a confocal microscope (Confocal Laser Microscope Leica SP8®).

**Radiolabeling of DTPA-SNs and NOTA-SNs with ⁶⁸Ga**

⁶⁸Ga (t½ = 68 min, β+ = 89% and EC = 11%) was obtained from a ⁶⁸Ge/⁶⁸Ga generator system (ITG Isotope Technologies Garching GmbH, Germany) in which ⁶⁸Ge (t½ = 270 d) was attached to a column based on an organic matrix generator. The ⁶⁸Ga was eluted with 4 mL of 0.05 M hydrochloric acid. Then, 500 µL of DTPA-SNs or NOTA-SNs (13 mg mL⁻¹) were mixed with 500 µL of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HEPES, 0.5 M, pH 5.05). The mixture was incubated with 1.5 mL of ⁶⁸Ga (=300 MBq) at 30 °C for 30 min and purified by PD-10 columns. The incorporated radiactivity was measured in an activimeter (AtomLabᴹᵀᴹ500, Biodex).

**Radiolabeling of NOTA-SNs, NOTA-HA-SNs and NOTA-PEG-SNs with ⁶⁷Ga**

⁶⁷Ga-citrate (t½ = 78.3 h, 100% EC = 39% γ 93 keV, 21% γ 185 keV, 17% γ 300 keV) was obtained from CURIUM (France) in a rotary vacuum evaporator to get a final volume of 500 µL. The pH was adjusted to 4–5 with NaOH 0.1 M, obtaining a solution of ⁶⁷GaCl₃ which was concentrated on an organic matrix generator. The ⁶⁷Ga⁺ ions were eluted with 3 mL of HCl 0.1 M, obtaining a solution of ⁶⁷GaCl₃ which was concentrated on a rotary vacuum evaporator to get a final volume of 500 µL. The pH was adjusted to 4–5 with NaOH 0.1 M and the solution was incubated with 500 µL of NOTA-SNs, NOTA-HA-SNs or NOTA-PEG-SNs (10 mg mL⁻¹) diluted in HEPES buffer (1.5 M, pH 5.05) for 1 h at 37 °C. The labeled nanoemulsions were eventually purified with PD-10 columns and the radioactivity was measured in the activimeter.

**Radiochemical Characterization**

The radiochemical yield (RCY) was calculated as a percentage of decay corrected activity found in the post-purification solution compared to the starting activity. The radiochemical stability (RCS) of ⁶⁷Ga-labeled nanoemulsions was assessed by incubating the emulsions with animal serum at 37 °C for 4 hours. In case of ⁶⁷Ga-labeled nanoemulsions, the stability was measured after incubation with animal serum for 0, 24, 48 and 72 h, according to the acquisition time points. In both cases, after the incubation time the mixture was purified by a PD-10 column and the activity of the elution was measured and decay corrected. Radiochemical purity (RCP) was analyzed by instant thin-layer chromatography (ITLC), and details regarding experimental protocols are included in the Supporting Information.

**In vivo Biodistribution by PET/CT and SPECT Imaging**

In vivo PET/CT imaging was performed in healthy mice (C57BL/6) with a nanoPET/CT small-animal imaging system (Mediso Medical Imaging Systems, Budapest, Hungary). List-mode PET data acquisition commenced 2 hours post bolus injection of ~12 MBq of ⁶⁷Ga-DTPA-SNs or ⁶⁷Ga-NOTA-SNs (12 MBq, n = 5) through the tail vein and continued for 30 minutes. At the end of PET, microCT was performed for attenuation correction and anatomic reference. The dynamic PET images in a 105×105 matrix (frame rates: 3×10 min, 1×30 min, 1×60 min) were reconstructed using a Tera-Tomo 3D iterative algorithm.

Acquisition and reconstruction were performed with proprietary Nucline software (Mediso, Budapest, Hungary). Qualitative Image analysis in mice was performed using Osirix software (Pixmeo, Switzerland). Animal experiments were conducted according to the ethical and animal welfare committee at CNIC and the Spanish and UE legislation. Experimental protocols have been approved by Madrid regional government (PROEX16/277).

SPECT studies were carried out on male Sprague–Dawley rats with an average weight of 299.5 ± 23.45 g supplied by the animal facility at the University of Santiago de Compostela (Spain). Planar dynamic SPECT images were acquired with a single-head clinical Siemens Orbiter gamma camera (Siemens Medical Solutions, Inc.,
USA) using a parallel collimator specifically designed for low-energy photons and high spatial resolution. Data were acquired in list-mode format in order to apply energy and spatial linearity, and uniformity corrections. In vivo NOTA-SNs and free ⁶⁷Ga biodistribution were studied after the intravenous injection (17.70 ± 8.5 MBq, n = 5) in healthy rats at different time-points: 24, 48 and 72 h. In order to compare the differences in biodistribution between NOTA-SNs, HA-SNs and PEG-SNs, healthy rats were intravenously injected (13.2 ± 0.3 MBq, n = 3) and the images were acquired dynamically during the first 60 min after injection (30 frames/2min). All images were analyzed using AMIDE software (amide.sourceforge.net). Quantitative analysis was carried out in a dynamic study by using circularly delineated Regions of Interest (ROIs) in the heart and liver, with 9 mm in diameter. The mean uptake was calculated over time in every region averaged over each of the 3 frames (6 min) and the results were reported as heart-to-liver ratio.

Ex vivo Biodistribution Studies
Ex vivo biodistribution of ⁶⁸Ga-labeled nanoemulsions was conducted 4 h post-injection. In case of ⁶⁷Ga-labeled nanoemulsions, biodistribution studies were performed 72 h post-injection. Animals were sacrificed in a CO₂ chamber, organs were extracted and counted with a Wizard 1470 gamma counter (Perkin Elmer) for 1 min each (n=5 per experiment). Radioactivity decay was corrected, and a biodistribution was presented as the percentage of injected dose per gram (% ID/g).

Statistical Analysis
All the experiments were performed at least in triplicate. Data are expressed as mean ± standard deviation (SD). Statistical analyses were calculated using GraphPad Prism® software (version 8.0). Student’s t-test was used to compare significant differences between the two groups. * (p ≤ 0.05), ** (p ≤ 0.01), *** (p ≤ 0.001) was considered statistically significant.

Results and Discussion
Preparation and Characterization of SNs, DTPA-SNs and NOTA-SNs
Here, we describe the radiolabeling of SNs with Gallium-68 and Gallium-67 for their application in PET and SPECT imaging. SNs were prepared by ethanol injection, a one-step mild technique that allows obtaining colloidal nanoemulsions within seconds (Figure 1A, left). The reproducibility of the preparation method (Figure 1A, right) was obtained after measuring 24 independent batches by DLS (raw data are showed in Table S1). SNs showed spherical morphology, as observed in FESEM images (Figure 1B). Additionally, SNs were efficiently internalized in cancer cells (Figure 1C), which is a relevant factor to take into account in order to determine the potential of a formulation for biomedicine applications. Stability determinations in cell culture media were also performed and are shown in Figure S1, Supporting Information. To convert SNs into suitable probes for PET and SPECT imaging, we followed a chelator-mediated approach, which is one of the most used methods to radiolabel nanoparticles with radionuclides, such as ⁶⁴Cu, ⁶⁸Ga, ⁹⁹mTc or ¹¹¹In. Labeling organic nanoparticles, and especially lipid nanoparticles, can be done by the use of lipid-derivative chelators. These conjugates can be inserted into the membrane of the lipid particles at the time of their preparation. In this study, we used two different lipid-derivative chelators to determine the best candidate for in vivo imaging. First, we selected the acyclic chelator diethylenetriaminopentaacetic acid modified with a dimyristoyl-sn-glycero-3-phosphoethanolamine chain (DTPA). Second, we synthesized the amphiphilic derivative of the macrocyclic chelator 1,4,7-triazacyclononane-1,4,7-triaacetic acid (NOTA) as previously described. The nucleophilic substitution in N,N-dimethylformamide afforded the corresponding thiourea derivative (2, NOTA-stearylamine, Figure S2A, Supporting Information) after recrystallization at moderate yield (23%). The NOTA-stearylamine derivative 2 was characterized by high-resolution mass spectrometry (Figure S2B Supporting Information) and NMR, confirming its structure (Figures S3 and S4 Supporting Information). Both lipid-modified chelators were spontaneously incorporated into the lipidic layer of SNs. According to results shown in Table 1, a slight increase in size was observed for DTPA-SNs and NOTA-SNs with respect to the control SNs, which could be indicative of the efficient incorporation of the chelators. In all cases, we observed a narrow distribution of the particles with a PDI ≤ 0.2. Stability studies under storage conditions at 4 °C showed that all the formulations were highly stable during the tested period (Figure 2A), indicating that the incorporation of the lipid-derivative chelators does not compromise the colloidal properties of SNs. In addition, they showed high stability in the human serum for 72 h at 37 °C, as shown in Figure 2B, demonstrating their potential for...
in vivo applications. Although organic nanoparticles offer relevant advantages with respect to inorganic nanoparticles, in general, their preparation is still complex, as dendrimers, liposomes or nanogels tend to require multi-step preparation methods and/or typically the use of high-energy techniques. On the contrary, this methodology provides long-term stable DTPA-SNs and NOTA-SNs particles in few minutes through a one-step protocol. Moreover, the preparation of DTPA-SNs and NOTA-SNs avoids the use of high-energy techniques and uses low-cost and conventional starting materials. In fact, compared with previously reported organic nanosystems, we describe here the simplest and easiest methodology for the gallium radiolabeling through a chelator-based strategy.33–35

Figure 1 (A) Scheme of the one-step method used for the preparation of SNs (left) and the method reproducibility after measuring the hydrodynamic size of 24 independent batches by DLS (right); horizontal bars represent size mean and standard deviation (127 ± 9 nm). (B) Representative Field Emission Scanning Electron Microscopy (FESEM) images of SNs acquired with STEM (top) and InLens (bottom) detectors. (C) Confocal microscopy images showing the internalization of SNs in different cancer cell lines. SNs are labeled in green (TopFluor-SM) and cell nuclei are labeled in blue (Hoechst).
Table 1 Physicochemical Characterization of SNs, DTPA-SNs and NOTA-SNs Measured by DLS and LDA (Results are Expressed as Mean ± Standard Deviation, n = 3)

| Formulation | Size [nm] | PDI       | Zeta Potential [mV] |
|-------------|-----------|-----------|---------------------|
| SNs         | 125 ± 1   | 0.14 ± 0.05 | −49 ± 5            |
| DTPA-SNs    | 136 ± 3   | 0.20 ± 0.02 | −51 ± 3            |
| NOTA-SNs    | 151 ± 7   | 0.15 ± 0.01 | −50 ± 5            |

Abbreviation: PDI, polydispersity index.

Radiolabeling of DTPA-SNs and NOTA-SNs for PET Imaging

The combination of nanomaterials with $^{68}$Ga for PET imaging has attracted a great deal of attention in recent years with several works devoted to the radiolabeling of inorganic nanoparticles. However, only a few studies with organic nanoparticles, specifically PEGylated DTPA and NODAGA liposomes, PAMAM dendrimer-DOTA conjugates, NODAGA and DOTA nanogels, NODAGA polymeric nanoparticles and PSMA-DOTA microemulsions, have been reported so far. DTPA-SNs, NOTA-SNs and non-chelator SNs (control) were radiolabeled by incubation with $^{68}$Ga$^{3+}$ at 30 °C for 30 min, and then purified by gel filtration in PD-10 columns. Figure 2C reveals that DTPA-SNs and NOTA-SNs were efficiently labeled with $^{68}$Ga, reaching RCY of 82 ± 4% for DTPA-SNs and 92 ± 2% for NOTA-SNs. Differences in RCY might be related to some release of $^{68}$Ga-DTPA-PE from the nanoemulsions in the purification process and/or to a better incorporation of the NOTA-SA derivative with SNs. With respect to the control formulation, nonspecific radiolabeling (RCY, 30 ± 12%) was observed. This might be due to some entrapment of the radioisotope into the lipid membrane of the nanoemulsions mediated by electrostatic interactions. Radiochemical yields were in line with other works in the field, such as liposomes, nanogels, biopolymer nanoparticles and microemulsions. The radiochemical purity was evaluated by ITLC using a sodium citrate solution as mobile phase. Under these conditions, free $^{68}$Ga showed a retention factor of 0.75 (Figure S5A, Supporting Information). In case of $^{68}$Ga-nanoemulsions, we could not detect the presence of free $^{68}$Ga, showing an RCP higher than 99% (Figure S5B, Supporting Information).
The RCS of the nanoemulsions upon incubation with serum at 37 °C was also determined (Figure 2D). As expected, radiolabeled control SNs (without a chelator) were not able to retain the gallium. In the case of DTPA-SNs, only 50 ± 3% of 68Ga were retained, while NOTA-SNs showed the highest stability, retaining 90 ± 2% of activity, again in line with cyclic chelators as NODAGA or DOTA.\(^{37,39,40,42}\) On the other hand, although DTPA is commonly used to form complexes with gallium and other radioisotopes, the formation of less stable complexes can be a consequence of its acyclic structure.\(^{43}\) This was confirmed after intravenous injection of DTPA-SNs and NOTA-SNs (12 MBq, n = 5) to healthy mice. 3D PET/CT images were acquired 2 h post-administration and showed major accumulation in the reticuloendothelial system (RES) organs and heart (Figure 3A). As expected, DTPA-SNs showed a higher circulation in the bloodstream due to the premature release of the radionuclide from the nanoemulsion. Ex vivo biodistribution results were conducted 4 h post-injection (Figure 3B) and corroborate major liver and spleen accumulation. This is in concordance with the in vivo pattern observed for most of the nanoparticles, especially lipid nanoparticles, such as liposomes and nanoemulsions.\(^{19,24,37,44}\) Remarkably, NOTA-SNs have a relatively long circulation time, showing a 10% of the injected dose in the bloodstream 4 h after intravenous injection. This in vivo pattern differs from other 68Ga-labeled emulsions recently reported, with a shorter circulation half-life, mainly due to differences in size and composition.\(^{41}\) These results indicate that the pharmacokinetics of NOTA-SNs can be better studied after the radiolabeling with longer half-life radioisotopes, such as 67Ga. Nevertheless, NOTA-SNs could be surface decorated with specific biomolecules in order to reduce their circulation time and to perform suitable probes for targeted 68Ga molecular imaging.\(^{45}\)

Radionuclide of NOTA-SNs with $^{67}$Ga for SPECT Imaging

$^{67}$Ga, compared with $^{68}$Ga (t½ = 68 minutes), allowed a long-term biodistribution study of NOTA-SNs by SPECT imaging. For NOTA-SNs radiolabeling, the clinical formulation $^{67}$Ga-citrate was initially converted into the chloride form ($\text{GaCl}_3$) as previously described.\(^{25}\) Briefly, $^{67}$Ga-citrates were trapped in a silica cartridge, washed with distilled water and finally eluted with HCl 0.1 M, rendering a 90% yield. To ensure a successful radiolabeling and taking into account that with the half-life of $^{67}$Ga there are no strong limitations for increasing the incubation time, we optimized the process (the solution was incubated with NOTA-SNs for 1 h at 37 °C). Then, the free radionuclide was removed by filtration in PD-10 columns, obtaining a 80 ± 2% of RCP (Figure 4A, before incubation with serum). The measured RCP was 98.9%, conducted by ITLC (Table S2, Supporting Information). In addition, RCS studies proved that the labeling was highly stable in serum over 72 h (Figure 4A). Then, due to the longer half-time of this radioisotope, SPECT studies were designed to evaluate the pharmacokinetics of NOTA-SNs at prolonged time periods. In vivo SPECT images were acquired 24, 48 and 72 h after intravenous injection of the radiolabeled NOTA-SNs. In parallel, we evaluated the ex vivo uptake of the free radioisotope as a control. Animals injected with free $^{67}$Ga (control) showed uptake in the bloodstream,
liver, lacrimal and salivary glands (Figure 4B), according
previous reports.34,47 We can also observe free 67Ga in the
bladder and kidneys, a consequence of renal clearance. In
comparison, NOTA-SNs showed similar biodistribution
than observed in PET/CT images, with main accumulation
in liver and RES organs, and its intensity decreases over
time. After 72 h, we measured the ex vivo biodistribution
and observed that the radioactivity remained only in liver
and spleen with less than 5% ID/g in both organs (Figure
S6, Supporting Information). This could be related to the
biodegradation of the particles and/or their excretion
through the urine, in line with our previous report in
which we described cationic fluorine-labeled nanoemulsions.24 However, further studies must be carried
out to determine the excretion routes of NOTA-SNs.

We finally investigated the possibility of surface-modifi-
cation of NOTA-SNs without interfering with the radiolabel-
ing procedure, to determine if it is possible to modulate the
in vivo behavior.48 Among the different strategies for surface
modification that have been reported to date, PEGylation is
the most established approach.49 We coated NOTA-SNs with
PEG (NOTA-PEG-SNs), using for this purpose a lipid-PEG
derivative. However, it is well known that PEGylation might
also lead to relevant drawbacks, such as the development of
an immunological response, antibody generation and toxic
side effects caused by the oxidative side products.50 Bearing
in mind these limitations, we have also investigated the
in vivo effect of an alternative coating, hyaluronic acid
(NOTA-HA-SNs). HA is a biocompatible polysaccharide
widely used in biomedical research that has been reported
to increase the circulating time of lipid nanoparticles.51,52
Surface-modified nanoemulsions (NOTA-PEG-SNs and
NOTA-HA-SNs) presented a similar size than the reference
formulation (NOTA-SNs) (Figure 5A). With respect to the
zeta potential, relevant modifications were only noticed in the
case of NOTA-HA-SNs, which rendered more negative
values (Figure 5B). Radiolabeling with 67Ga was success-
fully done, leading to RCY of 80 ± 8% and 76 ± 1% for
NOTA-HA-SNs and NOTA-PEG-SNs, respectively
(Figure 5C), indicating that the coatings do not significantly
interfere in the interaction between the chelator and the
radioisotope, and that coated NOTA-HA-SNs and NOTA-
PEG-SNs could be tracked by SPECT in a comparable fash-
on to the reference formulation (NOTA-SNs) (Figure S7,
Supporting Information).

A dynamic SPECT study was then carried out, and
the tracer uptake ratio between heart and liver was
calculated to evaluate the circulation/elimination phar-
macokinetic profile. As shown in Figures 5E, 67Ga-
P EG-NOTA-SNs showed significantly higher circulation
in the bloodstream in line with previous reports refer-
ing to PEGylated nanoemulsions.53 Noteworthy, the
PEGylation effect is observed even at a very low den-
sity (3 mol%), in concordance to other reports of nanoe-
mulsions in which PEG densities vary between 0.5 and
50 mol% with respect to the total amount of

Figure 4 (A) Radiochemical yield of 67Ga-NOTA-SNs (control) and radiochemical
stability after incubation with serum 37 °C at different points (0, 24, 48 and 72 h,
n=3). (B) Whole-body SPECT images showing the biodistribution of 67Ga-NOTA-
SNs compared with free 67Ga during 72 h (n=5).
In the case of NOTA-HA-SNs, the results were comparable to the reference formulation of NOTA-SNs, a fact that could be explained by the influence of the HA molecular weight and density of the coating. These factors will therefore need further optimization and will be the subject of future work intended for the development of applications in cancer nanotheranostics where HA coating could be particularly relevant to improve accumulation in the tumor.

Altogether, these results confirm that it is possible to modulate the composition and in vivo behavior of NOTA-SNs, to open up their application in specific indications in the biomedical field.

Conclusion

We described here a simple and highly efficient preparation method for chelator-functionalized biocompatible SNs and the subsequent radiolabeling with $^{68}$Ga and $^{67}$Ga. The radiolabeled formulations showed great radiochemical properties for in vivo applications and were efficiently followed-up by PET and SPECT imaging. Importantly, we have also proved that the biodistribution of the SNs can be modulated by modifying the surface properties. The capacity to modulate the radiolabeling, modality of imaging and tracking period, as well as the biodistribution properties, highlight the interest of SNs, which have the potential to be easily adapted to the requirements of different and specific biomedical applications. In summary, we believe that SNs have the potential for the development of advanced probes for nuclear imaging and nanotheranostics. In particular, future experiments could involve the evaluation of $^{68/67}$Ga-SNs in tumor bearing animal models to further determine the real potential of this formulation in cancer nanotheranostics, taking into account the anticancer properties of $^{67}$Ga.

Data Sharing Statement

All data generated or analyzed during this study are included in this published article and its Supporting Information file.
Ethics Approval and Consent to Participate

Animal experiments were conducted according to the ethical and animal welfare committee at CNIC and the Spanish and EU legislation. Experimental protocols were approved by Madrid regional government (PROEX16/277).

Author Contributions

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article will be submitted, gave final approval for the version to be published, and agreed to be accountable for all aspects of the work.

Funding

Authors thank the financial support given by Instituto de Salud Carlos III (ISCIII) and European Regional Development Fund (FEDER) (PI15/00828, PI18/00176 and DTS18/00133), by ERA-NET EURNOMANOMED III project METASTARG (AC18/00045) and by Asociación Española Contra el Cáncer (AECC, IDEAS18153DELAs). The first author also acknowledges the financial support from Axencia Galega de Innovación (GAIN) and Xunta de Galicia (IN848C_20170721_7) and ISCII (FI19/00206).

Disclosure

The author reports no conflicts of interest in this work.

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