Noninvasive imaging of radiolabeled exosome-mimetic nanovesicle using $^{99m}$Tc-HMPAO

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Exosomes known as nano-sized extracellular vesicles attracted recent interests due to their potential usefulness in drug delivery. Amid remarkable advances in biomedical applications of exosomes, it is crucial to understand in vivo distribution and behavior of exosomes. Here, we developed a simple method for radiolabeling of macrophage-derived exosome-mimetic nanovesicles (ENVs) with $^{99m}$Tc-HMPAO under physiologic conditions and monitored in vivo distribution of $^{99m}$Tc-HMPAO-ENVs using SPECT/CT in living mice. ENVs were produced from the mouse RAW264.7 macrophage cell line and labeled with $^{99m}$Tc-HMPAO for 1 hr incubation, followed by removal of free $^{99m}$Tc-HMPAO. SPECT/CT images were serially acquired after intravenous injection to BALB/c mouse. When ENVs were labeled with $^{99m}$Tc-HMPAO, the radiochemical purity of $^{99m}$Tc-HMPAO-ENVs was higher than 90% and the expression of exosome specific protein (CD63) did not change in $^{99m}$Tc-HMPAO-ENVs. $^{99m}$Tc-HMPAO-ENVs showed high serum stability (90%) which was similar to that in phosphate buffered saline until 5 hr. SPECT/CT images of the mice injected with $^{99m}$Tc-HMPAO-ENVs exhibited higher uptake in liver and no uptake in brain, whereas mice injected with $^{99m}$Tc-HMPAO showed high brain uptake until 5 hr. Our noninvasive imaging of radiolabeled-ENVs promises better understanding of the in vivo behavior of exosomes for upcoming biomedical application.

Exosomes are nano-sized extracellular vesicles which are produced by most of the cell types. Exosomes carry a subset of genetic materials and proteins to play key roles in delivery of the biological information between cells. Since exosomes were found initially to play a role in disposing unnecessary proteins such as transferrin receptor during reticulocyte maturation 30 years ago, a variety of functional roles have been elucidated. Especially, recently-alleged biological roles of exosomes ranged from their involvement in normal physiologic functions such as stem cell maintenance, immune modulation and tissue homeostasis to their contribution to the pathophysiology of several diseases. In addition, exosomes also delivered variable sets of bioactive materials specific for parent cell-types and mediated cellular activities of recipient cells.

Exosomes can be used as drug carriers by applying their role of in vivo intercellular communication. As natural endogenous nanoparticles, the autologous exosomes have the advantages of immune-tolerance and stability in circulation systems. In addition, exosomes derived from different cells have target-specific homing effect even to achieve active targeting. To enhance their targeting potency, ex vivo cell-derived

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Exosomes were engineered to carry specific surface proteins. According to these advantages, ex vivo cell-derived exosomes were tried to be used as nanocarriers. For example, exosomes were used to target cancer tissue by transporting therapeutic nucleic acids in animal experiments. Even in the clinical setting, cancer antigen-pulsed autologous exosomes isolated from patients were already applied for cancer immunotherapy. However, one major drawback of exosome-based nanocarriers was the small amount of natural exosomes. This low yield limits in vivo application where large amount of exosomes are necessary.

Recently, exosome-mimetic nanovesicles (ENVs) were collected from whole cells as alternatives to the natural exosomes also collected from the cells. This exosome-mimetic technology overcame several limitations of natural exosomes for the application of practical drug delivery, which included low yield (0.3 to 0.5 μg of exosomes per 10⁶ dendritic cells in 24 h) and difficulties in purification process. These ENVs have similar characteristics with natural exosomes in terms of size, morphology and protein contents. ENVs were easily produced with 100-fold higher amount than naturally collected exosomes, which enabled therapeutic application. In addition, ENVs shared the similar homing properties to the natural exosomes, which was revealed by showing accumulation of macrophage-derived ENVs in the tumors visualized by in vivo fluorescence imaging.

Considering that there is a variety of cell-type specific exosomes, in vivo behaviors of these exosomes may be highly varied. However, there were very few effective methods reported to investigate the in vivo behavior of these exosomes, which also became an obstacle for the biomedical application of these exosomes or ENVs. A breakthrough to investigate in vivo distribution and trace the exosomes is highly desired. To date, fluorescence imaging was tried to trace the whereabouts of the exosomes and to investigate the distribution of cell-derived membrane vesicles. Although in vivo optical imaging is a simple tool for in vitro and in vivo study, it has intrinsic limitations including difficulties in quantification and poor tissue penetration depth. Radioisotope (RI) labeling and nuclear imaging can easily overcome these limitations and can be applied to trace exosomes in vivo.

In this study, we developed a simple radiolabeling method for ENVs with ⁹⁹mTc-hexamethylpropyleneamine oxide (HMPAO) under physiologic condition, and visualized in vivo distribution of ⁹⁹mTc-HMPAO-ENVs using SPECT/CT. A scheme for radiolabeling of ENVs using ⁹⁹mTc-HMPAO was summarized in Fig. 1. ⁹⁹mTc-HMPAO is known as uncharged and highly lipophilic radiotracer, which has been widely used for cell labelling. It is well known that intracellular glutathione converts ⁹⁹mTc-HMPAO to the hydrophilic form to be trapped inside cells. Based on this property, ⁹⁹mTc-HMPAO has been clinically used for white blood cell (WBC) labeling for inflammation imaging. Since ⁹⁹mTc-HMPAO is an easily accessible radiotracer in the clinical setting, our approach is expected to facilitate biomedical application of exosomes by easily disclosing the whereabouts of exosomes or ENVs.

**Results**

**Radiolabeling of ENVs with ⁹⁹mTc-HMPAO.** ENVs produced from mouse Raw 264.7 macrophage cell line were purified from interface layer through Optiprep density gradient fractionation. When we measured glutathione in ENVs for ⁹⁹mTc-HMPAO labeling, we found 3.15 ± 0.16 nmol of glutathione in 0.1 mg of ENVs (Supplementary Fig. 1).

ENVs were incubated with ⁹⁹mTc-HMPAO for labeling under mild condition (in pH 7, PBS buffer) in order to prevent the labeled-ENVs from unexpected aggregation. After labeling, purification was performed in two methods to remove free ⁹⁹mTc-HMPAO, as described in Methods. Radiochemical purity was 99.6 ± 3.3% after the size exclusion column was applied (Fig. 2a), and 93.7 ± 5.4% after purification with exosome exclusive spin column with centrifugation. The procedure time of exosome-spine
column method was 3 min, which was 10 times less than the size exclusion column method (Table 1). Considering both methods showed comparable radiochemical purity while exosome exclusive spin column method took less time, the exosome-spin column method was used for further characterization study, SPECT/CT imaging and ex vivo biodistribution study.

Characterization of ENVs after $^{99m}$Tc-HMPAO radiolabeling. Size distribution of unlabeled ENVs and $^{99m}$Tc-HMPAO-ENVs was measured using nanoparticle tracking analysis (NTA). The average diameter of unlabeled ENVs was $218 \pm 8.2$ nm, and that of $^{99m}$Tc-HMPAO-ENVs was $213 \pm 6.8$ nm (Fig. 2b), showing no significant changes after $^{99m}$Tc-HMPAO radiolabeling. In order to confirm whether the exosome-specific protein contents of ENVs changed after $^{99m}$Tc-HMPAO radiolabeling, an exosomal marker, CD63, was measured by western blot analysis. Amount of exosomal protein in its expression was not changed, showing high expression of CD63 which was similar with protein expression in unlabeled ENVs (Fig. 2c). We performed the serum stability test for $^{99m}$Tc-HMPAO-ENVs. $^{99m}$Tc-HMPAO-ENVs showed comparable serum stability with that in PBS, maintaining around 90% until 5 hr (Fig. 2d).

In vivo SPECT/CT images and biodistribution of $^{99m}$Tc-HMPAO-ENVs. In vivo imaging of $^{99m}$Tc-HMPAO-ENVs and $^{99m}$Tc-HMPAO were acquired by SPECT/CT. Within 30 min after injection, $^{99m}$Tc-HMPAO-ENVs were taken up mainly by the liver and spleen (Fig. 3a). Of note, no significant brain
uptake was observed, compared with the significant brain accumulation in the mice with 99mTc-HMPAO-only administration at 30 min images (Fig. 3b). At 3 hr after the injection, 99mTc-HMPAO-ENVs accumulated in the salivary glands. In contrast, in the mice with 99mTc-HMPAO-only administration, radioactivity was relatively low in the liver and spleen, and this radioactivity decreased gradually until 5 hr (Fig. 3b). The intense uptake in the brain and intestine was observed immediately after the injection and the activity was remained until 5 hr. Salivary gland uptake was high in 99mTc-HMPAO-injected mouse group.

Table 1. Radiochemical purity and protocol time for each isolation method. *Procedure time: the time point until 99mTc-HMPAO-ENV was produced after the purification via size exclusion PD-10 column or exosome exclusive spin column. **Radiochemical purity: the measured % values by ITLC using Whatman no. 1 paper and 0.9% w/v NaCl solution as an eluent. Radiochemical purity was determined by following equation: E(%) = (1 – radioactivity of supernatant)/total radioactivity × 100%.

| Methods                           | Procedure time | Radiochemical purity (%) (n = 3/group) |
|-----------------------------------|----------------|---------------------------------------|
| Elution over PD-10 column         | 30 min         | 99.6±3.3                              |
| Centrifugation with exosome exclusive spin column | 3 min          | 93.7±5.4                              |

Figure 3. In vivo SPECT/CT images of 99mTc-HMPAO-ENVs injected in mice. After intravenous injection of 99mTc-HMPAO-ENVs or 99mTc-HMPAO, SPECT/CT images were acquired at 30 min, 3 hr, and 5 hr in BALB/c mice. (a) The SPECT/CT imaging showed the significantly intense uptake of 99mTc-HMPAO-ENVs in the liver and radioactivity in the salivary glands and intestine until 5 hr. (b) In contrast, high brain uptake and the delayed salivary glands uptake were observed in 99mTc-HMPAO-injected mouse group.

uptake was observed, compared with the significant brain accumulation in the mice with 99mTc-HMPAO-only administration at 30 min images (Fig. 3b). At 3 hr after the injection, 99mTc-HMPAO-ENVs accumulated in the salivary glands. In contrast, in the mice with 99mTc-HMPAO-only administration, radioactivity was relatively low in the liver and spleen, and this radioactivity decreased gradually until 5 hr (Fig. 3b). The intense uptake in the brain and intestine was observed immediately after the injection and the activity was remained until 5 hr. Salivary gland uptake was high in 99mTc-HMPAO-only administered group. Quantitative volume-of-interest analysis based on SPECT/CT images revealed similar distribution pattern to visual findings (Supplementary Fig. 2).

We additionally acquired SPECT/CT imaging of 99mTc-HMPAO-ENVs obtained from HB1.F3 human neural stem cells to test whether this method could be used in different type of cells. Just like the ENVs obtained from Raw 264.7 macrophage cells, the ENVs from neural stem cells showed the similar stability in the serum, and after systemic administration, they accumulated in the liver and spleen within 30 min and the uptakes in salivary glands increased gradually (Supplementary Fig. 3a,b). We also performed production of natural exosomes using Raw 264.7 macrophage cells and labeled them with the same method. After 3 weeks of culture and preparation, we could produce half the amount of exosomes than the ENVs, and SPECT/CT disclosed the distribution of 99mTc-HMPAO labeled natural exosomes. Low sensitivity due to lower amount of exosomes and radioactive label than ENVs made the images poor,
but accumulation pattern was similar to that of ENVs at 3 hr. 99mTc-HMPAO labeled natural exosomes accumulated in liver and spleen and then bowel (Supplementary Fig. 3c). Mice were sacrificed after 0.5, 1, 3, and 5 h of 99mTc-HMPAO-ENVs to investigate in vivo distribution using ex vivo quantitation of radioactivity. The distribution pattern was different particularly in the liver and brain between 99mTc-HMPAO-ENVs and 99mTc-HMPAO-only administered groups (Fig. 4).

Discussion
Exosome-based drug delivery receives great attention owing to merits as a naturally obtained nanoparticle which is able to bypass many issues of nanoparticles including immune activation, off-target effect and in vivo degradation6. Although cell-derived exosomes have showed therapeutic effects on specific inflammatory or cancer condition14,24, it remains unknown whether the injected exosomes are effectively delivered to the target tissue. To achieve breakthrough in exosome-based therapy, in vivo imaging is necessary.

In this study, we carried out radiolabeled-exosome imaging using ENVs which share biological features with naturally derived exosomes14. To investigate whereabouts and kinetics of administered ENVs or exosomes, imaging should be performed in living animals which can provide quantitative information even of the deep organs. Since exosomes are natural nanoparticles, distinct from artificially-made nanoparticles, labeling should be performed under biologically-inert and conservative condition. Until now, in previous studies fluorescence reporters or highly lipophilic dyes were used to track the in vitro and in vivo behavior in cells or living animals17,25,26. These methods had intrinsic limitations in light penetration depth and quantitation. MRI can overcome the disadvantage of optical imaging and modified liposomes were proposed as artificial exosomes which were constructed to contain surface markers and super paramagnetic labels for magnetic resonance imaging27, however, there was no suggestion how to make exosomes or ENVs with paramagnetic materials. Radionuclide labeling and imaging could be an alternative because of their high sensitivity, noninvasiveness and quantification of biodistribution. Moreover, ex vivo biodistribution study using radioactivity as we employed in this study accurately provides the absolute information of whereabouts after sacrifice of the animals even in the small organs with high sensitivity. To our knowledge, two studies have tried to develop radioisotope-labeled exosome to evaluate biodistribution, however, they only showed ex vivo biodistribution and a study engaged streptavidin reporter which needed genetically modified cells21,22. To achieve in vivo radionuclide-labeled exosome imaging under physiologic condition, we employed 99mTc-HMPAO. By reaction of 99mTc-HMPAO with sulfhydryl groups of the glutathione abundantly existing in cells, 99mTc-HMPAO became hydrophilic compounds and trapped within cells. Because of this property, ENVs composed of lipid bilayer were expected to be simply labeled with 99mTc-HMPAO under physiologic condition. This approach was introduced in glutathione-contained liposome labeling and was used for liposome imaging28,29. As liposomes and exosomes consist of lipid bilayer, and moreover, exosomes had glutathione inside the exosomes or ENVs, the 99mTc-HMPAO labeling resulted in comparable excellent radiochemical purity (higher than 95%). Furthermore, approximately 90% of entrapped 99mTc in ENVs persisted within the exosomes and ENVs in the serum until 5 hr. When erythrocytes and platelets were labeled with 99mTc-HMPAO, spontaneous release rate for 1 hr ranged from 5% to 10%10,31, which corresponded to that of ENVs. 99mTc-HMPAO labeling for ENVs could be performed under physiologic condition not to change their properties. In the in vitro study, CD63, an exosome marker, did not change after labeling. We also found that average size distribution did not change after labeling of ENVs with 99mTc-HMPAO. A small

Figure 4. Biodistribution of 99mTc-HMPAO-ENVs. Mice were sacrificed at 0.5, 1, 3, 5 hr after 99mTc-HMPAO ENVs injection and radioactivity was measured for quantitative analysis of biodistribution (n = 4 for groups sacrificed at 0.5, 1 and 5 hr; n = 3 for a group sacrificed at 3 hr). As shown in SPECT images, 99mTc-HMPAO-ENVs were highly accumulated in liver, spleen and kidneys. Note that no significant uptake in brain unlike 99mTc-HMPAO used as a brain perfusion tracer. %ID/g represents counts of radioactivity normalized by injected dose and weights of organ. Data are presented as mean ± S.D.
fraction of ENVs which were smaller than average size was identified after labeling (Fig. 2b), which could
mean that a small portion of ENVs would have been damaged during labeling procedures. Fortunately,
the overall size distribution was the same after the procedures. In the previous our work (unpublished),
ENVs were modified with SCN-Bn-NOTA, which is a bifunctional chelating agent, and then labeled
with 68Ga. After the 68Ga labeling, the size of ENVs increased up to 530 nm probably due to aggregations
which resulted in the accumulation in lung (Supplementary Fig. 4). We speculated that labeling condition
was closely related to aggregations and labeling for ENVs absolutely need the physiologic condition. In
this study, the size of 99mTc-HMPAO-labeled ENVs did not dramatically change as in 68Ga labeling after
labeling. In vivo SPECT/CT images of 99mTc-HMPAO-ENVs proved no lung accumulation suggesting
negligible aggregations during labeling procedures and in vivo after systemic injection.

In this study, we adopted two different purification methods based on either size exclusion column or
exosome exclusive spin column. Both methods showed comparable labeling efficiency with rapid radio-
labeling time, but in the case of size exclusion column method, large amount of ENVs was lost during
ebulation. In addition, the size exclusion column method takes 10 times longer until final elution than spin
column-based centrifugation (30 min vs. 3 min, Table 1). Although the loading volume of exosome in
spin column is limited (<100μl), we suggest that spin column-based centrifugation would be a better
method of radiolabeled ENVs isolation in terms of rapid and high yield of 99mTc-HMPAO-ENVs.

We found that 99mTc-labeled-ENVs could provide sensitive, quantitative and reproducible imaging. 99mTc-
HMPAO-ENVs administrated mouse showed high accumulation of injected ENVs in liver and spleen at 30 min, which was about 2–3 folds higher than 99mTc-HMPAO-only administered mouse. The
uptake of 99mTc-HMPAO-ENVs in the liver and spleen could be a result of phagocytosis in mononuclear
phagocyte systems. Since ENVs in our experiments were derived from mouse macrophage cells, they
could be actively captured by host-derived cells abundant in liver and spleen.25,26 SPECT/CT images of
99mTc-HMPAO-ENVs obtained from human neural stem cells revealed similar accumulation pattern to
those obtained from Raw 264.7 cells. Besides, similar-sized liposomes were reported to accumulate pas-
sively in mononuclear phagocyte systems9, and thus, the accumulation mechanism of ENVs might be
soon understood. Distribution study of natural exosomes was also feasible though the yield of exosomes
was not sufficient for radiolabeling and purification. To obtain SPECT/CT images of natural exosomes,
large amount of cells were needed to obtain minimum amount of natural exosomes. Despite this dif-
culty, we found that radiolabeled natural exosomes also showed similar distribution to radiolabeled
ENVs derived from macrophage and neural stem cells.

99mTc-HMPAO-ENVs imaging will be useful for in vivo drug-delivery monitoring. In biodistribu-
tion study, brain uptake was not observed in 99mTc-HMPAO-ENVs administration group, whereas
the 99mTc-HMPAO-only injected group showed high accumulation in the brain corresponding to pre-
vious study regarding in vivo kinetics of 99mTc-HMPAO34. Radioactivity of salivary glands and bowel
walls appeared at 3 hr and 5 hr in 99mTc-HMPAO-ENVs administration group like 99mTc-HMPAO-only
injected group, which might correspond to in vitro stability of 99mTc-HMPAO-ENVs, a result of eluted
99mTc-compounds from ENVs. Moreover, the release of 99mTc-compounds might be facilitated in vivo
due to phagocytosis in mononuclear phagocyte systems. These biodistribution patterns suggested
99mTc-HMPAO-ENVs can be used for tracing, particularly in early phase, considering that no brain
and minimal salivary gland uptake was detected. In the future, because of eluted 99mTc-compounds, accumu-
lation of ENVs in target tissue should be carefully interpreted as in 99mTc-HMPAO-labeled leukocyte
imaging in the routine clinical setting35.

As exosomes are derived from biological tissues, they potentially have advantages in clinical use for
diagnostic imaging as well as drug carriers. Many researchers have tried to exploit exosomes as poten-
tial drug delivery nanocarriers to transport chemical drug or siRNA to the disease site36. In spite of
the limitations in delayed spontaneous release of 99mTc-compounds, our approach has a wide range of
applications involving brain targeting, cancer and inflammation imaging. Radiolabeled-exosomes can be
used to track in vivo behavior of exosomes to find out where the administrated exosomes is localized
and how long exosomes is retained in the target tissue. Therefore, it can contribute to understanding
pharmacokinetics of the drug-loaded exosomes. Furthermore, in vivo imaging of exosomes derived from
several types of tissues under various environmental conditions in living subjects, enables to understand
in-depth biological properties and roles of exosomes. We expect that the radiolabeled-exosomes will be
gaining ground as a crucial imaging technique to apply exosomes to theranostics.

We reported a simple and rapid radiolabeling method of ENVs for in vivo radionuclide imaging,
and imaged biodistribution of 99mTc-HMPAO-ENVs via SPECT/CT in living animals. Highly lipophilic
99mTc-HMPAO which has been widely used for brain perfusion imaging agent was used for exosome
labeling with simple and rapid reaction. Though this was a feasibility study of SPECT/CT images for
exosomes, this approach has some limitations. Despite the high sensitivity of radiolabeled ENVs for
noninvasive imaging (7.4–14.8 MBq of 29–64μg radiolabeled exosomes for imaging as described in
Methods), labeling procedures can be improved more to reduce the loss of ENVs during labeling and
separation especially for low-yield natural exosomes although we showed the possibility of SPECT/CT
imaging of natural exosomes (Supplementary Fig. 3c). Furthermore, inhomogeneous vesicles derived
from cell organelles would have contaminated the ENVs as ENVs were produced by extrusion of cells
via polycarbonate membrane filters. Thus, further optimization of isolation techniques is warranted.
Nevertheless, in vivo distribution of 99mTc-HMPAO-ENVs was clearly observed by SPECT/CT, showing
Methods

Cell culture. Murine macrophage Raw 264.7 cell lines isolated from peritoneal fluid of BALB/c mouse was grown in Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen, Grand Island, NY). HB1.F3 human neural stem cells immortalized by retroviral transduction with the v-my c oncogene were grown in DMEM. All media were supplemented with 10% fetal bovine serum (FBS) and 10 U/mL penicillin and 10 μg/mL streptomycin (Invitrogen). All cells were cultured at 37°C in a humidified atmosphere of 5% CO₂. Cell culture reagents were purchased from Gibco-BRL.

Preparation of ENVs from cell lines. After adherent Raw 264.7 and HB1.F3 cells were detached through gentle scraping, cells were resuspended at a concentration of 5×10⁶ cells/mL using phosphate-buffered saline (PBS). The cell suspension was extruded three times through 10, 5, and 1.1 μm-sized polycarbonate membrane filters sequentially using amini-extruder (Avanti Polar Lipids). To make density gradient step, 50% iodixanol (Axis-Shield PoC AS, Oslo, Norway) was firstly placed at the bottom of an ultracentrifuge tube with gentle loading, and 10% iodixanol was overlaid in the second step. The extruded samples were finally loaded, and then the prepared samples were spun at 100,000×g for 2 h at 4°C using a Beckman Coulter type 45Ti rotor (Beckman Instruments, CA). ENVs were collected from the interface of the 50% and 10% iodixanol layers. And the produced ENVs were stored at −20°C.

Radiolabeling of ENV with ⁹⁹ᵐTc-HMPAO radiotracer. A commercially available kit of HMPAO was labeled with ⁹⁹ᵐTcO⁴⁻ without methylene blue. A vial containing 0.5 mg HMPAO and 4.0 pg SnCl₂ was reconstituted with 1110 MBq of ⁹⁹ᵐTcO⁴⁻ in 5 mL of 0.9% NaCl solution and shaken for 10 s. After 5 min of incubation, ⁹⁹ᵐTc-HMPAO was immediately added to the prepared ENVs. The ENVs (100 μg/100 μl) were incubated for 1 hr with prepared 185–370 MBq of ⁹⁹ᵐTc-HMPAO at room temperature. During the incubation, the tube was slowly shaken until the end of labeling.

Isolation of ⁹⁹ᵐTc-HMPAO-ENVs from free ⁹⁹ᵐTc-HMPAO. Two different methods were used to remove ⁹⁹ᵐTc-HMPAO from free ⁹⁹ᵐTc-HMPAO-ENVs. A size exclusion column or centrifugation with exosome exclusive spin column was used for purification of radiolabeled ENVs. For the size exclusion column, ⁹⁹ᵐTc-HMPAO-ENVs were isolated using PD-10 column (GE Healthcare, Milwaukee, WI, USA) eluted with 0.9% w/v NaCl solution. The eluent was collected in test tubes of each 0.5 mL fraction. Radiochemical purity was measured by thin-layer chromatography (TLC) using Whatman no.1 paper eluted with 0.9% w/v NaCl solution. The second, an exosome exclusive spin column with centrifugation was used (MW3000, Invitrogen). After the column was hydrated using 650 μl of 0.9% w/v NaCl solution, excess interstitial fluid was removed by spinning the column at 750×g for 2 min. The mixture of ENVs with ⁹⁹ᵐTc-HMPAO was centrifuged at 750×g for 2 min at room temperature to isolate ⁹⁹ᵐTc-HMPAO-ENVs. The radioactivity was examined using an AR-2000 TLC imaging scanner (Bioscan, Washington, DC).

Serum stability study for ⁹⁹ᵐTc-HMPAO-ENVs. ENVs (100 μg, 1 mg/ml in PBS) were incubated with 185 MBq ⁹⁹ᵐTc-HMPAO for 2 hr at room temperature. ⁹⁹ᵐTc-HMPAO-ENVs were immersed in PBS for 1.5 h to remove initial diffusion of lipophilic ⁹⁹ᵐTc-HMPAO from labeled-ENVs. ⁹⁹ᵐTc-HMPAO-ENVs were purified using exosome exclusive spin column. Samples loaded into 0.22 μm-filtered human serum were incubated for 10 min, 30 min, 1 hr, 3 hr, and 5 hr. The radiochemical purity at serum condition in TLC was analyzed using 0.9% w/v NaCl solution as an eluent. Radiochemical purity was expressed as percentage of counter of ⁹⁹ᵐTc-HMPAO-ENVs.

Nanoparticle tracking analysis. The size distribution of ENVs prepared by Raw 264.7 cells was measured by the nano tracking analysis (NTA) method using a NANOSIGHT NS500 (Malvern, Grovewood road, UK) by using minor modification of manufacturer’s instruction. Samples were diluted sufficiently for the contrast and minimal background level. The quick measurement mode was performed to find the optimal condition. Then, total 5 numbers of particle motion video were recorded automatically using standard measurement mode (temperature: 22.9°C and viscosity: 0.93 cP). The captured videos (5 videos per sample) were processed and analyzed (camera level: 4, capture duration: 60 s and detection threshold: 7). All other conditions were constant. The graphical figure was automatically drawn by software and given excel data was used for the mean value and the standard deviation.

Glutathione assay of ENVs. Total glutathione content of ENV prepared by Raw 264.7 cells was determined by glutathione assay kit (Cayman Chemical, Ann Arbor, NY) according to the instructions of the manufacturer. Briefly, 100 μg ENVs were resuspended and lysed in 0.1% Triton X-100 for 30 min. After 15 min centrifugation at 10,000×g at 4°C, the supernatants were reacted with DTNB (5,5’-dithiobis-2-nitrobenzoic acid) to produce yellow-colored TNB (-thio-2-nitrobenzoic acid) and the absorbance was read at 412 nm. To determine the concentration of glutathione, the reactions were carried
out with standard samples with known glutathione concentration. Three independent tests were carried out.

**Western blot analysis.** The concentration of prepared ENVs or $^{99m}$Tc-HMPAO-ENVs was quantified using BCA protein quantification kit (Thermo Scientific, MA). ENVs or $^{99m}$Tc-HMPAO-ENVs with equal amounts of protein were transferred onto PVDF membranes (Millipore, MA). PVDF membranes were blocked for 2 h at room temperature in Tris-buffered saline (TBS) containing 0.1% Tween20 and 5% nonfat dried milk. PVDF membrane was probed with primary antibodies against anti-rabbit CD63 (1:1000 dilution; Santa Cruz Biotechnologv, TX) at 4°C for overnight. PVDF Membranes were incubated with HRP-labeled anti-rabbit secondary antibody for 2 hr at RT, and immunocbescent detection was performed using chemiluminescence ECL detection system. Three independent experiments were performed in western blot assay.

**In vivo SPECT/CT imaging of $^{99m}$Tc-HMPAO-ENVs.** All animal experiments were conducted according to the protocols approved by the Institutional Animal Care and Use Committee at Seoul national university hospital, Seoul, Republic of Korea (Approval number: 13-0265-COA1). 8 week-old male BALB/c mice (Koatech, Seoul, Korea) were bred in a pathogen-free facility at Seoul National University Hospital Biomedical Research Institute. Weight-matched mice were housed in laboratory animal care facility in cages (5 mice/cage) and fed a standard diet. For isolation of $^{99m}$Tc-HMPAO-ENVs, the exosome exclusive spin column was used as aforementioned. 7.4–14.8 MBq of 29–64 μg $^{99m}$Tc-HMPAO-ENVs prepared by Raw 264.7 cells (n = 3) and 11.1 MBq of $^{99m}$Tc-HMPAO-ENVs prepared by HB1.F3 cells (30–35 μg) were also injected (n = 2). After the administration of $^{99m}$Tc-HMPAO-ENVs, serial SPECT/CT scans were acquired on a dedicated SPECT/CT scanner (NanoSPECT/CT, Bioscan, Washington, DC). All mice were anesthetized and maintained with 1.5% isoflurane at 1 L/min oxygen flow and placed on the prone position. The four multipinhole $\gamma$-detectors (9-pinholes) were used for high contrast collimation. Additional CT scans were acquired after SPECT acquisition for anatomical localization. Acquisition time was adjusted according to the counts to be more than 30,000 counts per projection. For SPECT images, 24 projections were obtained into an 80 × 80 acquisition matrix. The images were reconstructed by a 3-dimensional ordered-subsets expectation maximum (OSEM) algorithm. The radioactivity in the organs was calculated in the SPECT images by volume-of-interest analysis. Counts were corrected for the decay of radioisotope and normalized by percent injected dose per gram (%ID/g).

**In vivo biodistribution of $^{99m}$Tc-HMPAO-ENVs.** For biodistribution study, 74 kBq of $^{99m}$Tc-HMPAO-ENVs prepared by Raw 264.7 cells was injected into the tail vein of the mice (BALB/c, 7–8 weeks old, n = 15). Mice were sacrificed after 0.5, 1, 3, and 5 h. Heart, lung, skeletal muscle, liver, spleen, stomach, intestine, kidneys, brain, bone and blood samples were taken and weighed. The radioactivity of organs was determined by scintillation counting. The results were expressed as %ID/g.

**Preparation of natural exosomes and SPECT/CT images.** We extracted natural exosomes from Raw 264.7 cells. Complete DMEM cell medium was harvested after Raw 264.7 cells were incubated for 24 hours with exosome-depleted FBS prepared by ultracentrifugation at 150,000 G for overnight. Cell debris from harvested cell medium was removed by serial centrifugation at 3,000 g for 15 min and 3,000 g for 20 min. Natural exosomes were purified by ultracentrifugation at 150,000 G for 2 hours at 4°C. The collected exosome pellets were dissolved with PBS and the produced natural were injected into a BALB/c mouse and acquired SPECT/CT images as $^{99m}$Tc-HMPAO ENVs SPECT/CT at 3 hr after the injection.

**Ethics statement.** All animal experiments were conducted according to the protocols approved by the Institutional Animal Care and Use Committee at Seoul national university hospital, Seoul, Republic of Korea (Approval number: 13-0265-COA1).

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Author Contributions
D.S.L. and Y.S.G. designed the study, D.W.H., H.C., Y.S.L. and J.M.J. contributed to design the labeling scheme and perform radiolabeling procedures. D.W.H. and S.C.J. isolated exosome nanovesicles and H.C., N.E.C. and S.H. acquired and analyzed the in vivo image data. H.C., S.C.J., J.Y.P, H.J.O. and M.Y.Y. performed in vitro characterization study. D.W.H. and H.C. wrote this manuscript mainly and all other authors also wrote down their own part of this manuscript according to their own specialties.

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