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Radiolabeling optimization and characterization of ^{68}Ga labeled DOTA–polyamido-amine dendrimer conjugate – Animal biodistribution and PET imaging results

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Abstract

The present study describes the optimization of ^{68}Ga radiolabeling with PAMAM dendrimer–DOTA conjugate. A conjugate (PAMAM–DOTA) concentration of 11.69 μM , provided best radiolabeling efficiency of more than 93.0% at pH 4.0, incubation time of 30.0 min and reaction temperature ranging between 90 and 100 °C. The decay corrected radiochemical yield was found to be $79.47 \pm 0.01\%$. The radiolabeled preparation (^{68}Ga –DOTA–PAMAM–D) remained stable (radiolabeling efficiency of 96.0%) at room temperature and in serum for up to 4-h. The plasma protein binding was observed to be 21.0%. After intravenous administration, 50.0% of the tracer cleared from the blood circulation by 30-min and less than 1.0% of the injected activity remained in blood by 1.0 h. The animal biodistribution studies demonstrated that the tracer excretes through the kidneys and about 0.33% of the %ID/g accumulated in the tumor at 1 h post injection. The animal organ's biodistribution data was supported by animal PET imaging showing good 'non-specific' tracer uptake in tumor and excretion is primarily through kidneys. Additionally, DOTA–PAMAM–D conjugation with $\alpha_v\beta_3$ receptors targeting peptides and drug loading on the dendrimers may improve the specificity of the ^{68}Ga labeled product for imaging and treating angiogenesis respectively.

Graphical Abstract

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Keywords

Gallium-68 complexes; PAMAM conjugates; DOTA-NHS; Radiolabeling; Nanoparticles; Angiogenesis

Introduction

Dendrimers are highly branched, 3-dimensional polymeric structures which are often classified by the number of repeated branching cycles formed during synthesis and are reported to have an emerging role in a variety of biomedical applications (Tomalia and Frechet, 2002). These applications range from their use as biomimetic catalysts, drug carriers, gene delivery and boron neutron capture therapy (Huck et al., 1998; Peppas et al., 1994; Bielinska et al., 1996; Hawthorne, 1993). The dendrimer platform is considered potentially advantageous due to their relatively low immunogenicity, numerous surface functional groups and also their size, which is very close to various important biological polymers (Holister et al., 2010). Of the multitude of dendrimers that have been examined for biomedical applications, polyamidoamine (PAMAM) is the family of dendrimers that has been most extensively studied for their biomedical applications. These compounds are synthesized with polyamide branches functionalized with tertiary amines as focal points and an ethylene diamine core (Hawker and Frechet, 1990).

The current approach of linking the drugs with tumor specific molecular ligands like antibodies or receptor-specific ligands/peptides has inherent problem which lead to an inefficient binding due to the presence (of mutations) of diverse epitopic targets (Tobias et al., 2006; Wood et al., 2007). The concept of an enhanced permeability and retention (EPR) effect is now becoming the ‘gold standard approach’ for cancer targeting and drug designing. A wide range of nanotechnology products like fullerenes or dendrimers, macromolecular, polymeric and micellar particles including nanoparticles can exhibit ‘EPR’ effect for targeted therapeutic/drug delivery approach (Bharali et al., 2009; Greish, 2010). PAMAM dendrimers due to their enhanced permeability and retention properties may be used effectively both for drug loading and precise delivery to the angiogenic sites.

PAMAM dendrimers associated with 36 mer-anionic oligomers for delivering angiostatin and TIMP-2 genes have been reported to inhibit both tumor proliferation and angiogenesis

(Vincet et al., 2003). The gadolinium complexes of PAMAM dendrimers have been frequently studied as MRI contrast agents (Nwe et al., 2010; Bumb et al., 2010). However, complex of radio-metals with dendrimers in order to perform molecular imaging of angiogenesis has not been attempted.

The ability of PET imaging to detect higher percentage of positron emissions makes it two to three times more sensitive technique than the SPECT imaging (Rahmim and Zaidi, 2008). Of the radionuclides used in the clinical practice for PET, the use of Gallium-68 (half-life $t_{1/2} = 68$ -min; positron emission intensity 87%) is on the rise (Schultz et al., 2013b). Several identifiable properties of this radionuclide include superior image quality compared to SPECT radionuclides (*e.g.*, indium-111), the potential for an on-demand production *via* generator technologies that provide reliable and high-purity ^{68}Ga in sufficient quantities for routine radiopharmaceutical production without the need for cyclotron operations (Buchmann et al., 2007; Roesch, 2012). Generator technologies for ^{68}Ga production, chemistry of gallium, and emerging applications for ^{68}Ga radiopharmaceuticals have been reviewed in detail (Prata, 2012; Roesch, 2012). These physiochemical properties provide a strong basis to use PAMAM dendrimers with ^{68}Ga as a future PET tracer for imaging angiogenesis.

In the present study, tetraazacyclododecane tetraacetic acid mono (N-hydroxysuccinimide ester) (DOTA-NHS active ester) was conjugated to G4 PAMAM dendrimers. Purification and characterization of the conjugate was achieved and radiolabeling with ^{68}Ga was optimized. ^{68}Ga labeled product was subjected to pre-clinical evaluation through *in vitro* testing, cell toxicity, animal biodistribution and imaging studies.

2. Material and methods

2.1. Reagents

Reagents and chemicals of analytical grade used in the study were obtained commercially. Deionized ammonium acetate (1.0 M): methanol (1:1) was used as a mobile solvent for performing instant thin layer chromatography (ITLC). Sodium acetate trihydrate (Sigma-Aldrich, USA) was dissolved in 100.0 mL of water (Deionized Milli-Q) to prepare 0.2 M sodium acetate (NaOAc). To prepare buffer of the desired pH of 4.0, 9.0 mL of NaOAc (0.2 M) was mixed with 41.0 mL of 0.2 M acetic acid (HOAc). For cytotoxicity studies (MTT assay), the various reagents used in the study *e.g.* trypsin (0.25%, EDTA, 1.0 mM in PBS), - (4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (50.0 mg/mL, filter sterilized) and dimethylsulphoxide (DMSO) were procured from Sigma-Aldrich (USA). DOTA-NHS ester ($M_w = 761.48$ g/mole) and G4 PAMAM dendrimer ($M_w = 14,215$ g/mole) used in the present study were procured from Chematech, France and Sigma-Aldrich, USA respectively.

Matrix-assisted laser desorption/ionization (MALDI) (Ultraflexi, Bruker system) analysis was used for characterization of the conjugate and 2, 5-dihydroxybenzoic acid (2, 5-DHB) was used as a matrix for this technique. Sephadex G-25 medium (Sigma-Aldrich, USA) was used for the purification of DOTA conjugated G4-PAMAM dendrimer. ^{68}Ga was eluted using 0.05 M hydrochloric acid (HCl) from ^{68}Ge - ^{68}Ga generator (ITG, Munich, Germany). The starting activity for all the radiolabeling reactions was between 20.0 and 25.0 MBq.

Animal studies were carried out in adult male balb/c mice. The mice were maintained in the Central Animal House facility, PGIMER, Chandigarh, India and the study protocol was approved by the Institutional Animal Ethics and Bio-safety committees.

3. Methods

3.1. Conjugation

10.0 mg of G4 PAMAM dendrimers (703.0 nmoles) was added to 1.0 mL of NaOAc buffer (pH: 8.5) in a round bottom flask. DOTA-NHS ester (8.5 mg, 11,248.0 nmoles) was added in the flask and pH was adjusted to 7.5–8.0 with 1.0 M sodium hydroxide (NaOH). The mixture was stirred at room temperature for 48-h. The dendrimer–DOTA chelate was purified in Sephadex[®] G-25 (medium) using water as an eluent. The fractions were collected, lyophilized and dissolved in sodium acetate buffer for radiolabeling. The conjugate was diluted to 2.0 mg/mL in 0.1% trifluoro-acetic acid (TFA) for matrix-assisted laser desorption ionization time of flight-mass spectroscopy (MALDI-TOF-MS). An average number of DOTA molecules attached to the surface of PAMAM dendrimer were calculated as: [(increase in molecular weight relative to G4 PA-MAM dendrimer) ÷ (molecular weight of DOTA-NHS)].

3.2. Cytotoxicity assay (MTT assay)

In vitro cytotoxicity of DOTA–G4 PAMAM conjugate was evaluated by MTT assay. Exponentially growing cells were seeded at a uniform density of 5000 cells/well in a 96 wells microtitre plate 24 h before treatment. Cells were treated with varying concentrations (0.01–100.0 µg/mL) of the conjugate for three different time intervals (*i.e.* 24 h, 48 h and 72 h). At the end of the treatment, negative control and treated cells were incubated with MTT for 2 h at 37 °C. The medium was removed, 150.0 µL of DMSO was added to dissolve the formazan crystals and the optical density was measured at 570 nm (reference filter: 630 nm). Mitochondrial activity was expressed as percentage of viability of cells compared to the negative control.

3.3. Radiolabeling of ⁶⁸Ga with DOTA conjugated G4 PAMAM dendrimer

⁶⁸Ga was eluted from the ⁶⁸Ge/⁶⁸Ga generator in 5.0 mL of HCl (0.05 M). The various parameters like volume of the buffer used, pH, conjugate concentration and heating time of the reaction were standardized to achieve optimal radiolabeling of ⁶⁸Ga with DOTA–G4 PAMAM conjugate.

NaOAc buffer (0.2 M) at three different pH values (3.5, 4.0 and 5.0) was used for radiolabeling. 20.0 µL (~0.2 mg) of DOTA-dendrimer conjugate stock solution was added to the reaction vials containing 500.0 µL of buffer of varying pH followed by addition of 20.0–25.0 MBq of ⁶⁸Ga. The reaction mixture was stirred and incubated at 90–100 °C for 15–30 min. The radiolabeling efficiency of each mixture was analyzed by instant-thin layer chromatography (ITLC) using 1.0 M ammonium acetate:methanol (1:1) as a mobile solvent. Radiolabeling efficiency was evaluated as a function of varying buffer volume (50.0–1000.0 µL) and conjugate concentration (0.02–23.0 µM) while the pH of the reaction mixture was

kept constant at 4.0. All the above experiments were also performed at different incubation times of 5-min, 15-min, 30-min and 45-min.

3.4. Quality control tests of [^{68}Ga] DOTA–PAMAM-D

3.4.1. Radiolabeling efficiency—Briefly, 5.0 μL of the radiolabeled complex was applied at the marked origin point of the ITLC strip and the strip was placed in a solvent chamber at room temperature. The labeled complex moved to the solvent front while the free ^{68}Ga retained at the point of application. The retention factors (R_f) and the for the labeled compound and the free ^{68}Ga were calculated from the ITLC data. The percent radiolabeling efficiency of the radioligand was calculated.

3.4.2. In vitro stability—The *in vitro* stability of the radiolabeled formulation was done by measuring the radiolabeling efficiency (as described above) of the formulation at five different time intervals of 15-min, 30-min, 1-h, 2-h and 4-h.

3.4.3. Serum stability—The serum stability of radioligand was evaluated by incubating 0.8 mL of normal human serum with 0.2 mL of the radiolabeled complex in a vial at 37 °C. The percent radiolabeling efficiency of the radio-complex was estimated at incubation intervals of 15-min, 30-min, 1-h, 2-h and 4-h respectively. Any increase in free ^{68}Ga was considered as the degree of degradation.

3.4.4. Plasma protein binding—The *in vitro* protein binding of [^{68}Ga] DOTA–PAMAM-D was carried out in human plasma. About 0.9 mL of plasma sample was mixed with 0.1 mL of the radiolabeled compound and was incubated at 37 °C for 1-h. After 1-h incubation, 1.0 mL of 10% TCA was added to the mixture and centrifuged at 3000 rpm for 5-min. The supernatant was collected separately and the pellet was re-suspended in 10.0% TCA and centrifuged for 5-min. Radioactivity was measured in both the precipitate and supernatant fractions by using a well type gamma counter. The binding fraction of the total activity used was calculated and expressed as percent protein binding.

3.5. Tumor induction

Ehrlich's ascites tumor (EAT) cell lines were used to inoculate tumors in Balb/c mice (25–30 g). EAT cells were maintained in ascites form in the peritoneal cavity of mice, and were passaged weekly. Exponentially growing EAT cells were harvested, washed, and re-suspended in phosphate buffered saline (PBS). Tumors were induced by injecting about 1.5×10^7 cells subcutaneously in the fore limb of the mice. Tumor size was measured using a vernier caliper.

3.6. Blood kinetics and biodistribution studies

For conducting blood kinetics and animal biodistribution studies, 185.0 MBq radioactivity of ^{68}Ga labeled DOTA–PAMAM-D was used.

The blood kinetics and organ biodistribution studies were conducted in adult male balb/c mice (weight range 25.0–30.0 g). Briefly, 50.0 MBq radioactivity from a freshly prepared stock solution (185.0 MBq of ^{68}Ga labeled DOTA–PAMAM-D) was injected in the tail vein

of each mouse. Venous blood sample was collected from the ocular vein of each mouse at 3 different time intervals of 15-min, 30-min and 1-h. The radioactivity was counted in the blood and expressed as percentage of the injected dose per 1.0 mL of blood (% ID/mL).

Following radioactivity administration, normal and tumor bearing mice were sacrificed under light ether anesthesia at three different time points of 15-min, 30-min and 1-h. At each time point, three animals (9 in each group; total = 18) were used for biodistribution studies. Different organs *i.e.* like heart, lung, liver, kidney, spleen, intestine and brain were excised, washed in saline, dried and kept in pre-weighed test tubes. Tumor tissue was taken in case of tumor bearing mice. The tissues were weighed and radioactivity in each organ was counted. The results are presented as percent injected radioactivity per gram of tissue.

3.7. Positron emission tomography (PET) imaging of normal and tumor bearing mice

About, 37.0 MBq of [^{68}Ga] DOTA–G4 PAMAM dendrimer in 0.5 mL of normal saline was injected in tail vein of each normal and tumor bearing mouse. Mice were anesthetized by inhalation of 2.0% isoflurane in 2.0 L/min oxygen. A static whole body PET/CT image was acquired at 1 h post-injection by using animal hybrid imaging system (FLEX Triumph Regular FLEX X-O CT, LabPET4 Tri-modality system, GE Healthcare, Northridge, CA, USA; X-ray source: 70 kVp, 512 projections). All images were analyzed and reconstructed with VIVID (Amira, San Diego, USA).

4. Results

The MALDI analysis calculated the molecular weight (m/z) of the conjugate as 51,283. Further characterization of the conjugate revealed that on an average 48 DOTA molecules were attached to the surface of each unit of PAMAM-dendrimer.

MTT toxicity studies exhibited that up to conjugate concentration of 1.0 $\mu\text{g/mL}$, the cell viability is about 87.0% at 24 h. At higher concentrations of 10.0 μg and 100 μg (per mL), the corresponding values of cell viability are 66.0% and 59.0% respectively (Fig. 1).

The results of the ITLC-SG showing migration (peak indicating highest counts) of the radiolabeled compound ([^{68}Ga] DOTA–PA–MAM–D) and free ^{68}Ga (remaining at the origin) using 1.0 M ammonium acetate:methanol (1:1) as mobile phase are presented in Fig. 2.

The results demonstrating the effect of pH change (3.5–5.0) on the radiolabeling efficiency of [^{68}Ga] DOTA–PAMAM–D showed that the best radiolabeling efficiency of $96.8 \pm 0.01\%$ was observed at pH 4.0. There was a rapid fall ($\sim 68.0\%$) in the efficiency at pH 5.0. Likewise, using buffer volume of 500.0 μL yielded the best radiolabeling efficiency of more than 95.0% (Fig. 3).

Similarly, the effect of varying concentration of the conjugate (0.02–23.0 μM) on the percent radiolabeling efficiency was studied (Fig. 4). A conjugate concentration of 11.69 μM yielded highest radiolabeling efficiency (93.0%).

The best radiolabeling efficiency (96.0%) was achieved at 30-min of incubation time while keeping the reaction temperature constant between 90–100 °C (Fig. 5). The mean decay corrected radiochemical yield for all the experiments was found to be $79.4 \pm 0.01\%$.

In vitro and serum stability tests demonstrated that the radiolabeled product remained stable (with radiolabeling efficiency of >96.0%) for up to 4-h (Fig. 6). The plasma protein binding was observed to be $21.0 \pm 3.4\%$.

The blood pharmacokinetics studies demonstrated that following intravenous administration of [^{68}Ga] DOTA–PAMAM–D in mice, more than 50.0% the radiopharmaceutical cleared from circulation at 30-min and less than 1.0% of the radiotracer remained in blood at 1-h.

The normal animal biodistribution studies indicated that 2.0% of the injected dose (% ID/g) accumulated in kidneys at 30-min post administration. The radioactivity distribution in lungs, spleen and liver was lower than the kidneys. Heart and brain had shown very negligible uptake of the radiotracer. The results of the normal organ biodistribution studies are presented in Fig. 7.

Biodistribution results in tumor bearing mice showed that 0.33% of the ID/g accumulated in tumor tissue at 1 h post-injection (Fig. 8). The radiolabeled conjugate excreted mainly through kidneys.

The reconstructed animal PET/CT imaging data acquired at 1 h post administration indicated that there is minimal blood pool activity. And visualization of the kidneys and urinary bladder in the normal mouse explains that the radiotracer excretes primarily through the kidneys (Fig. 9a). The tracer showed good tumor uptake localized in the region of right forelimb (Fig. 9b).

5. Discussion

PAMAM dendrimers in the past have been studied extensively as prospective carriers for drug/gene delivery and moieties for modifying the drug solubility and absorption (Dong et al., 2010; Borowska et al., 2010). Additionally, dendrimer–metal chelator conjugation and subsequent complexation with metal ions have led to their use as effective MRI contrast agents (Brasch et al., 1997). However, radiolabeling of these conjugates as imaging probes has not been attempted so frequently. Recently, Kovacs et al. (2014) have attempted to radiolabel PAMAM dendrimers with $^{99\text{m}}\text{Tc}$ *via* hydrazinonicotinamide (HYNIC). These authors have reported the radiolabeling efficiency of 99.0% for $^{99\text{m}}\text{Tc}$ –PAMAM–HYNIC conjugate and the preparation remained stable for up to 24-h. These authors also showed that the radiolabeled product had rapid blood clearance and was excreted primarily through the hepatic and renal routes. However, SPECT imaging scores low over PET imaging in terms of sensitivity and image resolution (Rahmim and Zaidi, 2008).

The advances in generator technology for ^{68}Ga production, favorable chemistry of ^{68}Ga for radio complexation have paved the way for emerging applications of ^{68}Ga radiopharmaceuticals (Schultz et al., 2013a; Schultz et al., 2013b; Singh et al., 2013). The shorter half-life of ^{68}Ga (68-min), makes it compatible with the pharmacokinetics of most of

the low molecular weight radiopharmaceuticals (Nahhas et al., 2007). The most stable oxidation state of gallium in aqueous solution is +3 and its co-ordination number is 6. Ga(III) can undergo ligand exchange with transferrin once injected into the biological system. In order to avoid this, DOTA, a versatile chelator is widely used to bind large number of Ga(III) ions with high binding affinities during radiolabeling (Liu, 2008).

In the present study, we conjugated DOTA-NHS active ester with G4-PAMAM dendrimers and radiolabeling of the resultant conjugate with ^{68}Ga was optimized. Recently, Laznickova et al. (2014) have reported a successful radiolabeling of DOTA analog–G1/G4 PAMAM dendrimers conjugate with ^{177}Lu (beta emitting therapeutic radionuclide). These authors reported that G4 dendrimer conjugates modified on an average with 57 chelating units can be labeled with ^{177}Lu with high radionuclide purity and specific activity at 37 °C. In the present study, DOTA conjugated G4 PAMAM dendrimer was found to have on an average 48 chelating units on each dendrimer molecule. The preparation was radiolabeled with an efficiency of 96.0%. Factors like buffer pH and volume, concentration of conjugate and incubation time were optimized in order to achieve best radiolabeling efficiency. The lead for setting up the range of test conditions for optimizing the radiolabeling of PAMAM dendrimers with ^{68}Ga (group III-B metal ion) were obtained from previous studies (Biricova et al., 2011). These authors synthesized pyridine-N-oxide DOTA analog–PA-MAM dendrimers conjugate and radiolabeled the resultant product with ^{111}In and achieved a radiolabeling efficiency of about 95.0%. The pH of buffer plays an important role in radiolabeling procedures especially with ^{68}Ga and the reaction kinetics for the incorporation of Ga^{3+} is inversely related to pH (Bartholoma et al., 2010). We observed the best radiolabeling efficiency at pH 4.0.

The observed radiolabeling efficiency for the synthesis of [^{68}Ga] DOTA–PAMAM-D was comparable as has been reported for ^{68}Ga labeled peptides previously (Maecke et al., 2005). The rapid clearance of ^{68}Ga labeled PAMAM-D from the blood was in agreement with the previous studies (Malik et al., 2000; Roberts et al., 1996). A significant amount of radioactivity accumulated in kidneys which indicated the excretion of this conjugated system *via* kidneys. Biricova et al. reported a high uptake of ^{111}In –DOTA analog–PAMAM dendrimer in kidneys immediately after radio- activity administration in rats.

Animal PET imaging data showed that maximum tumor to background ratio was obtained at 1 h post injection of [^{68}Ga] DOTA–PAMAM-D suggesting that the designed nanoprobe can efficiently target tumor tissues and can be retained at tumor site due to enhanced permeability and retention (EPR) effect. Sadekar et al. (2011) reported that branched PAMAM dendrimer (G-6) showed significantly higher accumulation in ovarian tumor bearing mice than the conventional linear N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer of comparable molecular weight. The use of radiolabeled dendrimers due to their topology, functionality and dimensions has been described as a promising approach for the molecular visualization of tumor angiogenesis (Maeda et al., 2001).

The present study describes that G-4-PAMAM dendrimers could be conjugated successfully with bi-functional chelate (DOTA) and the subsequent radiolabeling with ^{68}Ga could be achieved with high radiolabeling efficiency and stability. Pre-clinical animal studies showed

that the radiolabeled conjugate could accumulate and retain in the tumor tissue through passive targeting and that the radiolabeled dendrimers could be excreted through kidneys.

The potential of radiolabeled DOTA–PAMAM dendrimers for the PET based detection of tumor angiogenesis shall be further explored by conjugating $\alpha_v\beta_3$ receptors targeting peptides to the DOTA–PAMAM-D conjugate which may further improve the specificity of the ^{68}Ga labeled product for imaging angiogenesis.

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HIGHLIGHTS

- Chemical conjugation of G-4 PAMAM dendrimers with DOTA-NHS carried out successfully.
- Purification and characterization of the conjugate was done by SEC and MALDI-TOF.
- Radiolabeling of PAMAM-DOTA-conjugate with ^{68}Ga yielded high radiolabeling efficiency.
- [^{68}Ga] DOTA-PAMAM-Dhas rapid blood clearance and excreted mainly through the kidneys.
- No significant retention of the radiotracer was seen in any other organ.

Fig. 1.
Cytotoxic effects of DOTA-PAMAM-D in BMG cells studied by % cell viability using MTT assay.

Fig. 2.
ITLC-SG of the [⁶⁸Ga] DOTA-PAMAM-D: mobile phase 1 M ammonium acetate:methanol (1:1).

Fig. 3.
Radiolabeling efficiency (%) of $[^{68}\text{Ga}]$ DOTA-PAMAM-D calculated at different volumes of buffer.

Fig. 4. Radiolabeling efficiency (%) of [^{68}Ga] DOTA-PAMAM-D at different concentrations of conjugate.

Fig. 5.
Radiolabeling efficiency (%) of $[^{68}\text{Ga}]$ DOTA-PAMAM-D with different heating times.

Fig. 6.
In vitro and serum stability of [^{68}Ga] DOTA-PAMAM-D as a function of time.

Fig. 7. Biodistribution pattern of $[^{68}\text{Ga}]$ DOTA-PAMAM-D in various organs of normal balb/c mice at three different time intervals (15 min, 30 min and 60 min).

Fig. 8.
Biodistribution pattern of $[^{68}\text{Ga}]$ DOTA-PAMAM-D in various organs of tumor bearing balb/c mice at three different time intervals (15 min, 30 min and 60 min).

Fig. 9.

(a) Reconstructed PET/CT imaging in normal Balb/c mice with [^{68}Ga] DOTA-PAMAM-D.

(b) Reconstructed PET/CT imaging in EAT bearing Balb/c mice with [^{68}Ga] DOTA-PAMAM-D.