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Radiosynthesis, biodistribution and micro-SPECT imaging study of dendrimer–avidin conjugate

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ABSTRACT

Partially acetylated generation five polyamidoamine (PAMAM) dendrimer (G5-Ac) was reacted with biotin and 2-(*p*-isothiocyanatobenzyl)-6-methyl-diethylenetria minepentaacetic acid (1B4M-DTPA), respectively to form the complex Bt-G5-Ac-1B4M which was further conjugated with avidin to give the conjugate Av-G5-Ac-1B4M. Then both of the conjugates were radiolabeled with technetium-99m (^{99m}Tc), respectively. Their in vitro cellular uptake study shows that the conjugate of Av-G5-Ac-1B4M-^{99m}Tc exhibits much higher cellular uptake in HeLa cells than that of Bt-G5-Ac-1B4M-^{99m}Tc. Accordingly the following evaluation such as in vitro/in vivo stability, biodistribution and micro-SPECT imaging was observed only for the conjugate of Av-G5-Ac-1B4M-^{99m}Tc.

1. Introduction

Cancer has been one of the major social and health concerns for the last 10 decades, although the milestones have already achieved in both of diagnosis and therapeutics. The applications of nuclear medicine in oncology are of a particular importance as a rapidly developing therapeutic and diagnostic multimodality. Radiopharmaceuticals are dosage forms consisting of two components, a carrier and a tracer amount of a radionuclide with a defined radiation types. Therefore the efficacy of radiopharmaceuticals is determined by both of the two components.[1]

The advantages of conjugating small molecule drugs to water-soluble, nontoxic, biocompatible polymers have been repeatedly documented[2–4] which includes improved drug solubility, long blood circulation time, decreased toxicity, and possibility to deliver drug payloads specifically to disease sites via both passive[5–7] and active targeting methods.[8,9] Within this area, the dendritic polymer architecture holds significant potential due to a number of decisive advantages.[10–14]

Dendrimers are a new class of highly branched spherical polymers that are highly soluble in aqueous solution and have a unique surface of many functional groups. Compared with many other types of already synthesized dendritic macromolecules, dendrimers are generally monodisperse and structural/chemical uniformity, which is critical for drug delivery applications. Polyamidoamine (PAMAM) dendrimers with huge amine groups have the advantage of being able to be conjugated easily to other molecules via an amide linkage, which is one of the most fundamental and widespread chemical bonds in nature.[13,14]

Technetium-99m (^{99m}Tc) is so far the most commonly used radionuclide in Single Photon Emission Computed Tomography (SPECT) imaging. More than 80% of all usually used radiopharmaceuticals contain the short-lived metastable radionuclide.[15] This is due to the highly interesting physical properties of ^{99m}Tc among with short half-life (6 h) and gamma photon emission of 140 keV, which is advantageous for both effective imaging and patient safety perspectives. ^{99m}Tc can be derived from ^{99}Mo/^{99m}Tc generator as column elutes which makes it readily available. Furthermore ^{99m}Tc possesses latent chemical properties which facilitating thereby the labeling of several types of kits for versatile diagnostic applications.

Avidin is a quickly internalizing molecule into either normal hepatocytes or cancer cells, especially ovarian and colorectal adenocarcinoma cells, which expresses β-D-galactose receptors[16–20] and extremely easy to conjugate with biotin. Recently we reported radiolabeled dendrimer folic acid conjugate has certain accumulation in KB tumor tissues, meanwhile much higher concentration of the conjugate was observed in the folate receptor positive tumors when the dendrimer folic acid conjugate was PEGylated via both of in vivo biodistribution and micro-SPECT imaging study.[21,22] However the conjugate containing folic acid molecule has much

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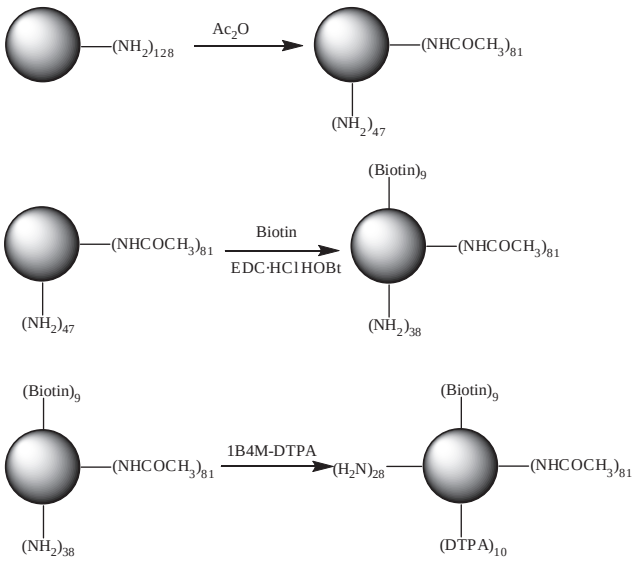
accumulation in kidneys, so we tried to employ avidin instead of folic acid to observe the biodistribution and micro-SPECT imaging. Herein we report the synthesis, characterization, and preliminary biological evaluation of ^{99m}Tc radiolabeled acetylated dendrimer–avidin conjugate.

2. Results and discussion

2.1. Syntheses of dendrimer–avidin conjugate

To decrease the toxicity and non-specific cellular uptake,[23,24] the primary amine groups on the surface of PAMAM dendrimer were partially converted to acetamide moieties in the presence of acetic anhydride and triethylamine. The degree of acetylation was measured by ^{1}H NMR, using the ^{1}H proton integration method. ^{1}H NMR spectrum of the acetylated dendrimer showed the proton signal at d 2.36 ppm, which corresponded to the methylene protons of $-CH_2C(O)-$ in G5 PAMAM dendrimer, The specific signal at d 2.00 ppm corresponded to the methyl protons of induced acetyl groups. The integration ratio of these two kinds of proton signals in the acetylated dendrimer suggests that an average of 81 acetyl groups is present on the surface of each G5 PAMAM dendrimer (G5-Ac$_{81}$).[21,25] G5-Ac$_{81}$ was further biotinylated in which the reaction is active with biotin catalyzed by 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide HCl (EDC·HCl) and 1-hydroxybenzotriazole (HOBt). The avidin/HABA assay revealed that on average of 9.5 biotin molecules were conjugated to each G5-Ac$_{81}$ molecule (Bt$_9$-G5-Ac$_{81}$) which was further confirmed by the ^{1}H NMR spectrum. The heterocyclic bridge hydrogen of biotin proton peaks could be observed at 4.44 and 4.62 ppm. From the integral ratio of the biotin proton at 4.44 and 4.62 ppm to the methylene protons of $-CH_2C(O)-$ in dendrimer PAMAM G5, approximately 9.5 biotin molecular were found attached to each molecule of PAMAM dendrimer. For detecting the conjugates in live animals, we employed ^{99m}Tc as radioactive nuclide, which is commercially available and easy to coordinate with bifunctional chelating agent DTPA. The partially acetylated dendrimer was reacted further with 1B4M-DTPA, whereas isothiocyanates are active enough to react easily with terminated primary amine of PAMAM, and the degree of functionalization can be controlled by stoichiometric control of reagents ratio and determined by a similar manner above. Therefore the final product containing 81 acetyl, 9.5 biotins and 9.75 1B4M-DTPA which was defined as Bt$_9$-G5-Ac$_{81}$-1B4M$_{10}$ (Scheme 1).



Scheme 1. Synthetic procedure for Bt-G5-Ac-1B4M conjugate.

The conjugate Bt$_9$-G5-Ac$_{81}$-1B4M$_{10}$ gave excellent radiochemical yield (95.6%) which can be used directly without further purification (Fig. 1b). A high performance liquid chromatograph (HPLC) equipped with a radioactivity γ-detector was used to monitor the conversion. The HPLC chromatograms in Figure 1a show that Na^{99m}TcO$_4$ had a retention time of 5 min, and the radiolabeled products were shown in the Figure 1b. Through the comparison of the two charts and our previous study[21,22], we could deem the Bt$_9$-G5-Ac$_{81}$-1B4M$_{10}$-^{99m}Tc had a retention time of 11.25 min.

The radiolabeled conjugate of Av-G5-Ac$_{81}$-1B4M$_{10}$-^{99m}Tc was given efficiently from Bt$_9$-G5-Ac$_{81}$-1B4M$_{10}$-^{99m}Tc and avidin due to its extremely high affinity of avidin to biotin (K_d = 10^{-15} M)[26,27] (Scheme 2).

2.2. Evaluation of in vitro/in vivo stability

After we got the radiolabeled conjugate Av-G5-Ac$_{81}$-1B4M$_{10}$-^{99m}Tc, we evaluate its stability in vitro and in vivo. The results were shown in Figure 2. Within 6 h about 93% and 78% of the conjugate still keeps the original structure in PBS solution and newborn calf serum at 37 °C, respectively. In vivo at least 78% of conjugate keeps good stability within 6 h in healthy mice.

2.3. Cell internalization assay

The concentration of conjugates is 500 nM. In this concentration, the toxicity is very low and likely to be ignored.[23,24]

The human ovarian cancer HeLa cells showed progressive internalization of the radioactivity when incubated at 37 °C (Fig. 3), and 56% of the radioactivity of Avidin-G5-Ac$_{81}$-1B4M$_{10}$-^{99m}Tc was internalized after 8 h of incubation. In contrast, HeLa cells showed no significant internalization of radioactivity for Bt$_9$-G5-Ac$_{81}$-

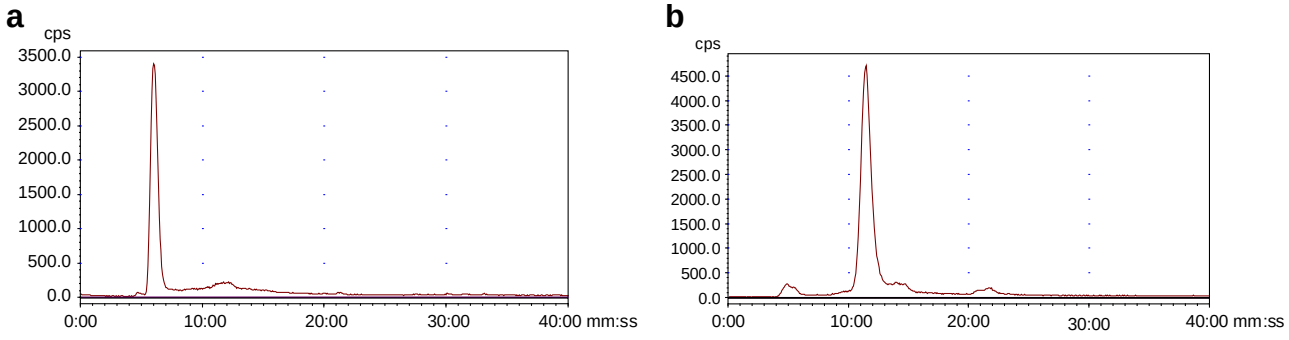
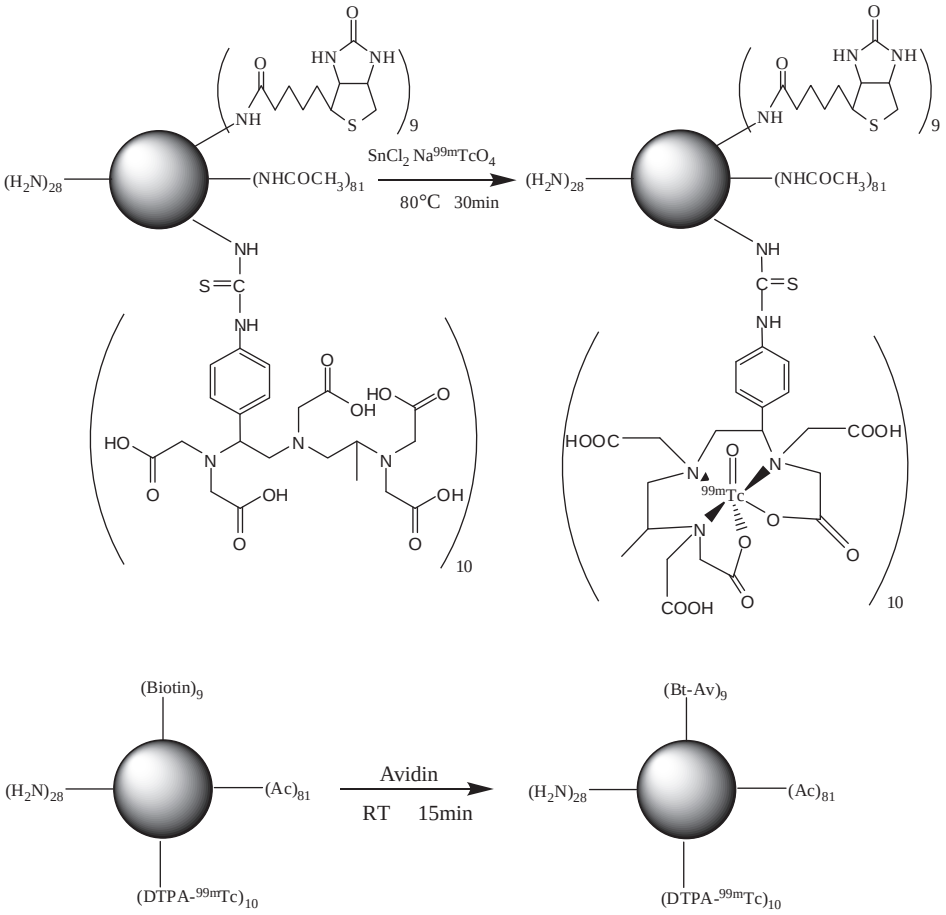


Figure 1. High performance liquid chromatography (HPLC) analyses of the complex (radioactivity γ-detector): (a) Na^{99m}TcO$_4$; (b) Bt$_9$-G5-Ac$_{81}$-1B4M$_{10}$-^{99m}Tc.



Scheme 2. Radiolabeled procedure for Av-G5-Ac-1B4M-^{99m}Tc conjugate.

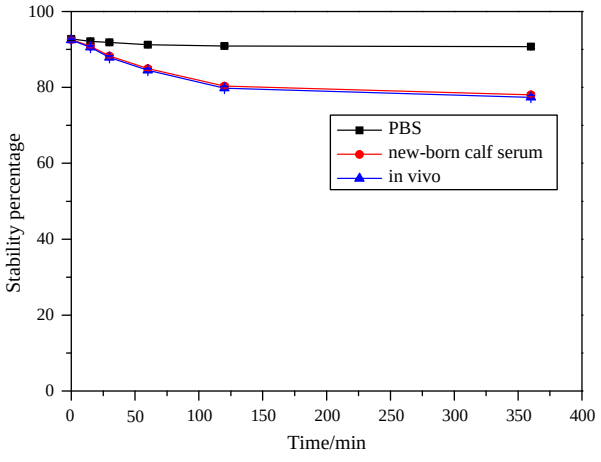


Figure 2. In vitro/in vivo stability of Av-G5-Ac$_{81}$-1B4M$_{10}$-^{99m}Tc conjugate.

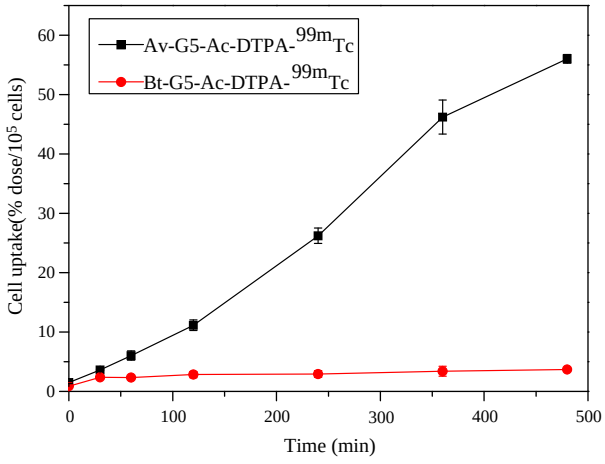


Figure 3. Time-dependent cell uptake of avidin-G5-Ac$_{81}$-1B4M$_{10}$-^{99m}Tc and Bt$_9$-G5-Ac$_{81}$-1B4M$_{10}$-^{99m}Tc to HeLa cells (error bars: $n = 3$).

1B4M$_{10}$-^{99m}Tc. After 8 h of incubation with Hela cells, less than 4% of the radioactivity of Bt$_9$-G5-Ac$_{81}$-1B4M$_{10}$-^{99m}Tc was internalization.

The result that the Av-G5-Ac$_{81}$-1B4M$_{10}$-^{99m}Tc cell uptake of HeLa cell is much higher than Bt$_9$-G5-Ac$_{81}$-1B4M$_{10}$-^{99m}Tc revealed that avidin has certain target to HeLa cell, because avidin contains terminal *N*-acetylglucosamine and mannose residues that bind some lectins which are expressed at various levels on the surface of many cancer cells such as ovarian cancer.[17,19,28,29]

2.4. Pharmacokinetics and biodistribution study

We next evaluated the pharmacokinetic blood clearance of the radiolabeled compounds. Thus the normal healthy mice received an intravenous dose of 0.74 MBq of the radiolabeled compound, and blood samples were collected at various time intervals thereafter. As shown in Figure 4, the conjugate was rapidly removed from circulation in the mouse. The distribution phase half-life $t_{1/2}(\alpha)$ of the radiolabeled conjugate was estimated to be 1.1 min

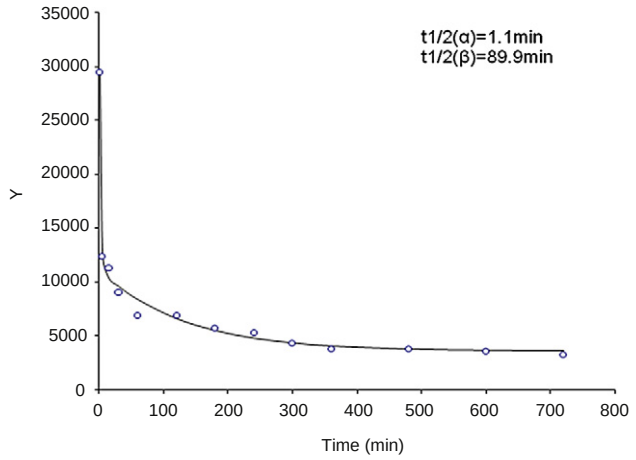


Figure 4. Concentration–time curve of avidin-G5-Ac_{81}-1B4M_{10}-^{99m}Tc.

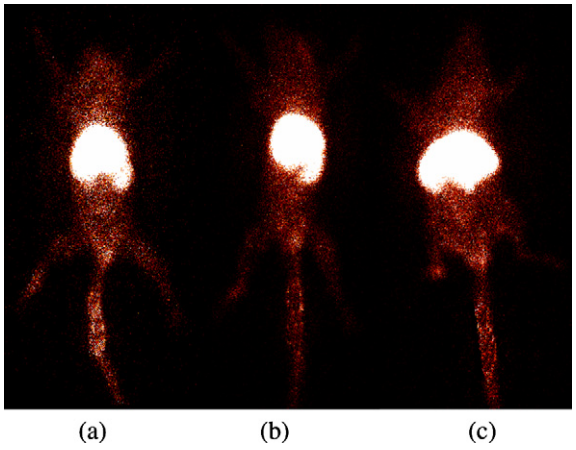


Figure 5. Micro-SPECT imaging of normal healthy mouse at (a) 2 h, (b) 4 h and (c) 6 h.

and the clear-phase half-life $t_{1/2}(\beta)$ was estimated to be 89.9 min. $t_{1/2}(\alpha)$ = 1.1 min illustrates that the conjugate could rapidly distributed throughout the body, $t_{1/2}(\beta)$ = 89.9 min reveals that the conjugate could clear fast from blood. These properties reveal the Avidin-G5-Ac_{81}-1B4M_{10}-^{99m}Tc has the potential to be an ideal body tracer.

The in vivo biodistribution was assessed by normal healthy Kunming mice. After receiving the same dose of labeled compound Av-G5-Ac_{81}-1B4M_{10}-^{99m}Tc, animals were euthanized at the designated times and selected tissues were removed, weighed, and counted to determine ^{99m}Tc distribution. The percentages of ID/g in normal mice at 2 h, 4 h and 6 h post-injection of Av-G5-Ac_{81}-1B4M_{10}-^{99m}Tc were shown in Table 1.

The concentration for radiolabeled compound Av-G5-Ac_{81}-1B4M_{10}-^{99m}Tc was cleared rapidly from blood which was further confirmed by the data of pharmacokinetic assay. However the compound showed very high accumulation in liver and spleen. Splenic accumulation may be attributable to uptake by the reticulo-endothelial system. In regard to the hepatic retention of this complex, two points should be described. One of them is related to the positive net charge of the complex, which could drive some interaction with hepatic cells, facilitating the process of internalization (non-specific).[16,30,31] The other one is related to the presence of mannose residues in the molecule of avidin that interacts specifically with respective receptors presented on hepatocytes.[16,32] The conjugate Av-G5-Ac_{81}-1B4M_{10}-^{99m}Tc may contain superabundant avidin, so it accumulation in liver very high. We also observed that the compound showed higher uptake in the lung tissue. It is possible that the resultant compound had become extremely large and was trapped in the microvascular bed in the lung. Mamedea et al. reported that intravenously injected ^{111}In-oligo/G4-bt-$Av_{25,50,100}$ complexes showed extremely high accumulation in the lung. They speculated that the size of these complexes led to passive deposition in the vascular bed.[16] In our study, after conjugation with avidin, the complex maybe large enough to be trapped in the lung.

In the other organs, such as kidney, the uptake of the conjugate kept at low level which is quite different from that of the dendrimer folic acid conjugate.[20,21] The concentration in the brain was very low at all time points which suggest that the dendrimer conjugate could not cross the blood–brain barrier.

Table 1
Biodistribution of avidin-G5-Ac_{81}-1B4M_{10}-^{99m}Tc at 2, 4 and 6 h in normal mice

Organs	2 h	4 h	6 h
Blood	1.44 ± 0.05[a]	0.91 ± 0.21	0.77 ± 0.02
Heart	2.48 ± 0.22	2.38 ± 0.75	1.24 ± 0.01
Liver	45.55 ± 2.14	47.74 ± 1.33	56.54 ± 1.30
Spleen	43.80 ± 0.50	47.38 ± 2.00	51.09 ± 5.99
Lung	12.56 ± 0.89	9.39 ± 1.81	7.31 ± 2.92
Kidney	2.12 ± 0.17	2.88 ± 0.01	3.35 ± 0.40
Intestine	0.84 ± 0.18	0.45 ± 0.15	0.44 ± 0.06
Stomach	0.82 ± 0.07	0.51 ± 0.01	0.48 ± 0.05
Muscle	0.24 ± 0.01	0.18 ± 0.05	0.16 ± 0.02
Skin	0.69 ± 0.26	0.57 ± 0.12	0.51 ± 0.08
Bone	0.67 ± 0.02	0.55 ± 0.09	0.47 ± 0.01
Brain	0.04 ± 0.01	0.04 ± 0.02	0.05 ± 0.01

[a] Values are shown as mean ± SD (ID/g%) (n = 3).

2.5. Micro-SPECT imaging

The predominant uptake of Av-G5-Ac_{81}-1B4M_{10}-^{99m}Tc by liver and spleen was further confirmed by micro-SPECT imaging. As shown in Figure 5 a ventral image taken of a mouse at 2 h, 4 h, and 6 h after receiving 18.5 MBq dose of distinctively localizes the γ-radiation to the liver and the spleen, meanwhile no appreciable radiotracer was observed in kidney. Our preliminary result suggests that we may find some radiolabelled conjugate which avoid the disadvantage of the dendrimer folic acid conjugate.

3. Conclusion

The radiolabeled conjugate of Avidin-G5-Ac_{81}-1B4M_{10}-^{99m}Tc was successfully prepared and characterized which exhibits excellent in vitro/in vivo stability and rapid clearance from blood. The in vitro cell uptake assay revealed that the conjugate for Avidin-G5-Ac_{81}-1B4M_{10}-^{99m}Tc could bind efficiently to HeLa cell, both of the in vivo biodistribution and micro-SPECT imaging study show that the high uptake was observed in liver and spleen while low in kidney. The further evaluation on tumor bearing nude mice is under way and will be reported in due course.

4. Experimental section

4.1. Materials and methods

PAMAM dendrimer generation five (PAMAM-G5), biotin (Bt), triethylamine and 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide HCl (EDC·HCl, 98%) 1-hydroxybenzotriazole (HoBt, 99%) were purchased from Aldrich Co., Ltd. Acetic anhydride, dimethyl sulfoxide (DMSO, 99%) and dialysis membrane (MWCO, 3500 and

14,000) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai). Carrier-free ^{99m}Tc-pertechnetate was freshly eluted with saline from ^{99}Mo/^{99m}Tc-generator (Shanghai Yuanpu Isotope Technology. Co, Ltd). 2-(*p*-Isothiocyanatobenzyl)-6-methyl-diethylenetria minepentaacetic acid (1B4M-DTPA) was a gift from Dr. Martin W. Brechbiel (NIH). All of the reagents were used as received without further purification. HeLa cells were purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Kunming mice was obtained from Shanghai Laboratory of Animal Center, Chinese Academy of Sciences. ^{1}H NMR spectrum was performed on Bruker AVANCE DRX 500 spectrometer in D_2O solution. The micro-SPECT imaging system developed in our institute consists of a stationary mini γ camera with pinhole collimator or parallel collimator and vertical object rotation mechanism. The mini γ camera is based on a 5 mm thick pixellated NaI (T1) crystal array with 1.2 mm pixel size and 1.4 mm pixel pitch coupled to a five in diameter Hamamatsu R3292 Position Sensitive Photomultiplier Tube (PSPMT).

4.2. Reverse phase high performance liquid chromatography

A Phenomenex (Torrance, CA) Jupiter C5 silica-based HPLC column (250 mm × 4.6 mm, 300 Å) was used for the separation of analyst. Two Phenomenex Wide pore C5 guard columns (4 mm × 3 mm) were also installed upstream of the HPLC column. The mobile phase for elution of PAMAM dendrimers was a linear gradient beginning with 90:10 water/acetonitrile (ACN) at a flow rate of 1 mL/min, reaching 50:50 after 30 min. Trifluoroacetic acid (TFA) at 0.14 wt % concentration in water as well as in ACN was used as a counterion to make the dendrimer conjugate surfaces hydrophobic. The conjugates were dissolved in the mobile phase (90:10 water/ACN). The injection volume in each case was 100 μL with concentration of approximately 1 mg/mL. The contained radioactive outflow of fluid was collected in a sealed vial.[20,21]

4.3. Syntheses

4.3.1. Synthesis of partially acetylated PAMAM dendrimers (G5-Ac)

Partially acetylated G5 PAMAM dendrimer (G5-Ac) was synthesized according to a reported method.[21] Briefly, acetic anhydride (75% ratio of primary amine numbers of a G5 PAMAM dendrimer) was added slowly to the G5 dendrimer solution (1.39 mmol G5 dendrimer dissolved in 4 mL methanol) in the presence of triethylamine (1.25 equivalents of acetic anhydride). The mixtures were then stirred under N_2 atmosphere for 24 h at room temperature. Acetic acid, a byproduct of the acetylation, was removed by extensive dialysis (MWCO = 3500 Da) of the reaction mixture against PBS buffer and double-distilled water for 3 days. The obtained sample G5-Ac was lyophilized and stored in a dry place before further modification and characterization. Yield: 93.8%. ^{1}H NMR (500 MHz, D_2O), δ 2.00 (244 H, s), 2.36 (504 H, br).

4.3.2. Synthesis of Bt-G5-Ac from biotin and acetylated PAMAM dendrimers

Firstly 0.015 g biotin was dissolved in 5 mL dimethyl sulfoxide (DMSO) in a 50 mL round bottom flask then 0.024 g of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC·HCl) and 0.018 g 1-hydroxybenzotriazole (HOBt) was added and mixed for 1 h at room temperature to give active ester. Then 5 mL DMSO solution containing 0.1 g of G5-Ac dendrimer was added to the mixture and stirred for an additional 24 h under nitrogen atmosphere. The resulting mixture was purified by dialysis (using cellulose membrane with 14,000 MWCO, PBS buffer, and DI water three times, respectively with 1 L for each time) and lyophilization. The obtained compound is the conjugate of Bt-G5-Ac, which was lyophilized and stored in a dry place before further modification. Yield: 94.5%. ^{1}H NMR (500 MHz, D_2O), δ: 2.00 (244 H, s), 2.36 (504 H, br), 4.44 (9 H, s), 4.62 (10 H, s).

4.3.3. Measurement of biotinylation rate with 4-hydroxyazobenzene-2-carboxylic acid (HABA) assay

Briefly the avidin/HABA reagent was prepared according to the manufacturer's instructions by adding 10 mg of avidin and 600 mL of 10 mM HABA (Sigma) to 19.4 mL PBS (pH = 7.4). Hundred milli liters of serially diluted Bt-G5-Ac solution was added to 900 mL of the avidin-HABA solution, and the absorbance was measured at 500 nm.[22,23]

4.3.4. Synthesis of Bt-G5-Ac-1B4M from 1B4M-DTPA and Bt-G5-Ac

The Bt-G5-Ac conjugate was concentrated to the concentration of 5 mg/mL and reacted with excess of 1B4M-DTPA (MW = 555 g/mol) at 40 °C, and the ratio of Bt-G5-Ac to 1B4M-DTPA was 1:20 (molar ratio). The reaction mixture was maintained at pH 9–10 during the reaction time of 36 h. The resulting mixture was purified by dialysis (using cellulose membrane with 14,000 MWCO, PBS buffer, and DI water 3 times respectively with 1 L for each time) and lyophilization. The obtained compound is the conjugate of Bt-G5-Ac-1B4M, which was lyophilized and stored in a dry place before further modification. Yield: 97.5%. ^{1}H NMR (500 MHz, D_2O), δ: 2.00 (244 H, s), 2.36 (504 H, br), 4.44 (9 H, s), 4.62 (10 H, s), 6.84–6.88 (18 H, m), 7.16–7.30 (21 H, m).

4.3.5. ^{99m}Tc radiolabeling of conjugate Bt-G5-Ac-1B4M to give Bt-G5-Ac-1B4M-^{99m}Tc

[^{99m}Tc] sodium pertechnetate was eluted from a ^{99m}Tc/^{99}Mo generator using 0.9% saline. Na[^{99m}TcO4] (5 mCi in 1 mL of saline) was added to the shielded vial containing the Bt-G5-Ac-1B4M solution (20 mg in 2 mL of helium-purged water, contained 0.1 mL 1 M of stannous chloride). The reaction vial was purged with nitrogen, shaken, and heated for 30 min in a water bath (80 °C) before analysis. The final reaction mixture was analyzed by RP-HPLC.[20]

4.3.6. Synthesis of Av-G5-Ac-1B4M-^{99m}Tc from avidin and Bt-G5-Ac-1B4M-^{99m}Tc

According to the reported method,[24] the labeled conjugate was mixed with avidin (the molar ratio of Bt-G5-Ac-1B4M-^{99m}Tc to avidin was 1:18) and incubated for 15 min at room temperature.

4.4. Evaluation of in vitro/in vivo stability

The in vitro stability of the synthetic complex was studied by measuring the radiochemical purity using radio-HPLC (the conditions of the RP HPLC assay was used to measure the stability were as same as described above) at different time intervals after preparation. The complex was added to a test tube containing PBS solution. The mixture was incubated by shaking at 37 °C in a thermomixer. The radiochemical purity was measured at 30 min, 1 h, 2 h, 4 h and 6 h by radio-HPLC. The same procedure was applied to the experiment using newborn calf serum.

The in vivo stability was evaluated as followed, after preparation of the labeled compound, 1.85 MBq conjugate was withdrawn and 100 μL of saline was added. Then the conjugate solution was injected into healthy Kunming mice (4 weeks old) via tail vein. Mice were euthanized and blood was removed quickly after certain times (15 min, 30 min, 1 h, 2 h, 4 h, 6 h) (n = 3 for all time points). 0.1 mL HCl (6 M) was added to the blood samples, which were centrifuged at 10,000 rpm for 1 min at 20 °C, and the supernatant was collected in a test tube containing CH_3CN (500 μL). After the tube was vortexed for 30 s and centrifuged at 10,000 rpm for 5 min at

20 °C, the supernatant was withdrawn to measure the radiochemical purity by RP-HPLC.

4.5. Cell internalization assay

HeLa cell lines (human uterine cervix carcinoma cells) were obtained from Shanghai Cell Repository of Chinese Academy of Sciences. Cells were maintained in RPMI 1640 (Gibico) with 10% inactivated fetal bovine serum (Gibico), penicillin (100 IU/mL) and streptomycin (100 lg/mL), and L-glutamine. The cell lines were grown in logarithmic growth at 37 °C in a humidified atmosphere consisting of 5% CO_2 and 95% air.

The HeLa cells were harvested using 0.25% trypsin-EDTA and seeded 1×10^5 cells per well of a 24-well plate and incubated for 12 h, The cells were then incubated with the conjugates (final concentrations of 0.037 MBq for Bt-G5-Ac-1B4M-^{99m}Tc and Av-G5-Ac-1B4M-^{99m}Tc, the concentration of conjugates is 500 nM) and maintained at 37 °C for 30 min, 1 h, 2 h, 4 h, 6 h and 8 h. At the end of incubation period, the medium was moved, the cells were washed three times with 0.5 mL PBS buffer, the medium (including PBS buffer) and cells was collected, and the radioactivity was measured with a γ-counter. The percentage of cell uptake radioactivity of total radioactivity was calculated.

4.6. Pharmacokinetics and biodistribution study

The pharmacokinetics was determined in normal healthy Kunming mice (4 weeks old). Each animal received a dose of 0.74 MBq of the radiolabeled compound Av-G5-Ac-1B4M-^{99m}Tc in approximately 0.2 mL volume via the tail vein ($n = 5$). At the designated times (1, 5, 10, 15, 30, 60, 120, 240, 360 min), each animal's blood was immediately collected via the tail vein. The blood clearance curve for in normal mice was detected in a γ-counter. The $t_{1/2}(\alpha)$ and $t_{1/2}(\beta)$ were calculated using Drug and Statistics Software DAS 2.0 (Wuhu Gauss Data Analysis Ltd) where C concentration is the amount of radioactivity per milligram (cpm/mg) in blood of normal mice.

For further study of nude mice, we first evaluated the biodistribution of conjugate Av-G5-Ac-1B4M-^{99m}Tc in normal healthy mice. The normal healthy Kunming mice were intravenously via the lateral tail vein with the radiolabeled compound ($n = 3$ at all time points), Mice were sacrificed at various time points (2, 4 and 6 h). Their organs were harvested and weighed, and their radioactivity was measured in a γ-counter and counted to determine the percentage of activity incorporated into the tissues. Each time (data) point was carried out in quintuples which were expressed as percentage of injected dose per gram of tissue (% ID/g ± SD).

4.7. Micro-SPECT imaging

Meantime we make use of Micro-SPECT to evaluate the imaging ability of Av-G5-Ac-1B4M-^{99m}Tc. Also we select the normal healthy Kunming mouse. The mouse was injected 18.5 MBq of Av-G5-Ac-1B4M-^{99m}Tc (the concentration of Av-G5-Ac-1B4M was 500 nM) through the tail vein. Five minutes planar static images in prone were acquired at 2 h, 4 h and 6 h after injection, using a γ-camera equipped with a pinhole collimator.

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References and notes

1. Misara, H.; Muhammad, A. K.; Roudayna, D.; Hatem, F. *Adv. Drug Delivery Rev.* **2008**, *60*, 1329.
2. Park, J. H.; Lee, S.; Kim, J.; Park, K.; Kim, K.; Kwon, I. C. *Prog. Polym. Sci.* **2008**, *33*, 113.
3. Kopecek, J.; Kopeckova, P.; Minko, T.; Lu, Z. R.; Peterson, C. M. *J. Controlled Release* **2001**, *74*, 147.
4. Jensen, K. D.; Nori, A.; Tijerina, M.; Kopeckova, P.; Kopecek, J. *J. Controlled Release* **2003**, *87*, 89.
5. Matsumura, Y.; Maeda, H. *Cancer Res.* **1986**, *46*, 6387.
6. Maeda, H.; Seymour, L. W.; Miyamoto, Y. *Bioconjugate Chem.* **1992**, *3*, 351.
7. Maeda, H.; Wu, J.; Sawa, T.; Matsumura, Y.; Hori, K. *J. Controlled Release* **2000**, *65*, 271.
8. Khandare, J.; Minko, T. *Prog. Polym. Sci.* **2006**, *31*, 359.
9. Lu, Z. R.; Shiah, J. G.; Sakuma, S.; Kopeckova, P.; Kopecek, J. *J. Controlled Release* **2002**, *78*, 165.
10. Frechet, J. M. J. *Science* **1994**, *263*, 1710.
11. Bosman, A. W.; Janssen, H. M.; Meijer, E. W. *Chem. Rev.* **1999**, *99*, 1665.
12. Frechet, J. M. J.; Tomalia, D. A. *Dendrimers and Other Dendritic Polymers*; John Wiley & Sons Ltd: West Sussex, 2001.
13. Frechet, J. M. J. *J. Polym. Sci., Part A: Polym. Chem.* **2003**, *41*, 3713.
14. Tomalia, D. A. *Prog. Polym. Sci.* **2005**, *30*, 294.
15. Banerjee, S.; Pillai, M. R.; Ramamoorthy, N. *Semin. Nucl. Med.* **2001**, *31*, 260.
16. Mamedea, M.; Sagaa, T.; Ishimori, T.; Higashi, T.; Sato, N.; Kobayashi, H.; Brechbield, M. W.; Konishia, J. *J. Controlled Release* **2004**, *95*, 133.
17. Yao, Z.; Zhang, M.; Sakahara, H.; Saga, T.; Nakamoto, Y.; Sato, N.; Zhao, S.; Arano, Y.; Konishi, J. *Ann. Nucl. Med.* **1998**, *12*, 115.
18. Yao, Z.; Zhang, M.; Sakahara, H.; Nakamoto, Y.; Higashi, T.; Zhao, S.; Sato, N.; Arano, Y.; Konishi, J. *J. Nucl. Med.* **1999**, *40*, 479.
19. Yao, Z.; Zhang, M.; Sakahara, H.; Saga, T.; Arano, Y.; Konishi, J. *J. Natl Cancer Inst.* **1998**, *90*, 25.
20. Kobayashi, H.; Sakahara, H.; Hosono, M.; Yao, Z. S.; Toyama, S.; Endo, K.; Konishi, J. *J. Nucl. Med.* **1994**, *35*, 1677.
21. Zhang, Y. Q.; Sun, Y. H.; Xu, X. P.; Zhu, H.; Huang, L. L.; Zhang, X. Z.; Qi, Y. J.; Shen, Y. M. *Bioorg. Med. Chem. Lett.* **2010**, *20*, 927.
22. Zhang, Y. Q.; Sun, Y. H.; Xu, X. P.; Zhang, X. Z.; Zhu, H.; Huang, L. L.; Qi, Y. J.; Shen, Y. M. *J. Med. Chem.* **2010**, *53*, 3262.
23. Jevprasesphant, R.; Penny, J.; Jalal, R.; Attwood, D.; McKeown, N. B.; D'Emanuele, A. *Int. J. Pharm.* **2003**, 263.
24. Kolhatkar, R. B.; Kitchens, K. M.; Swaan, P. W.; Ghandehari, H. *Bioconjugate Chem.* **2007**, 2054.
25. Yang, W. J.; Cheng, Y. Y.; Xu, T. W.; Wang, X. Y.; Wen, L. P. *Eur. J. Med. Chem.* **2009**, *44*, 862.
26. Green, N. M. *Adv. Protein Chem.* **1975**, *29*, 85.
27. Kobayashi, H.; Kawamoto, S.; Saga, T.; Sato, N.; Ishimori, T.; Konishi, J.; Ono, K.; Togashi, K.; Brechbiel, M. W. *Bioconjugate Chem.* **2001**, *12*, 587.
28. Lotan, R.; Raz, A. *Ann. N. Y. Acad. Sci.* **1988**, *551*, 385.
29. Hama, Y.; Urano, Y.; Koyama, Y.; Kamiya, M.; Bernardo, M.; Paik, R.; Krishna, M. C.; Choyke, P. L.; Kobayashi, H. *Neoplasia* **2006**, *8*, 607.
30. Girão, T.; Simões, S.; Pires, P.; Nir, S.; Pedroso de Lima, M. C. *Biochim. Biophys. Acta* **2001**, *1510*, 136.
31. Boussif, O.; Lezoualc'h, F.; Zanta, M. A.; Mergny, M. D.; Scherman, D.; Demeneix, B.; Behr, J. P. *Proc. Natl. Acad. Sci.* **1995**, *92*, 7297.
32. Nag, A.; Ghosh, P. C. *J. Drug Target.* **1999**, *6*, 427.