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Synthesis and Preliminary Evaluation of a ^{99m}Tc-labeled Folate-PAMAM Dendrimer for FR Ifmaging

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Abstract

Folate receptor is an ideal target for tumor-specific diagnostic and therapeutic. The aim of this study is to synthesize ^{99m}Tc-labeled folate-PAMAM dendrimer modified with 2-hydrazinonicotinic acid (^{99m}Tc-HP$_3$FA) for FR imaging. The ^{99m}Tc-HP$_3$FA conjugate was prepared using

N-tris-(hydroxymethyl)-methylglycine and trisodium triphenylphosphine-3,3',3''-trisulfonate as co-ligands. Physicochemical properties, *in vitro* cell uptake study and *in vivo* micro-SPECT/CT imaging were performed. The radiolabeled ^{99m}Tc-HP$_3$FA conjugate was prepared with high radiolabeling yield, good stability and water-solubility (logP=-1.70±0.21). In cell uptake study, the radiolabeled conjugate showed high uptakes in the FR-abundant KB cells, and could be blocked significantly by excess folic acid. The 7721 cells which served as control group substantially had no uptakes. The results of micro-SPECT/CT imaging exhibited that high accumulation of activity was found in FR overexpressed KB tumor, and the tumor to muscle ratio was approximately 25.78. While, using free FA as inhibitor, the uptakes of ^{99m}Tc-HP$_3$FA in KB tumor and kidney were obviously inhibited. In summary, a new radio-compound was synthesized successfully with specifically FR targeting ability. The feasibility of ^{99m}Tc-HP$_3$FA for early diagnosis of FR-positive tumors with noninvasive single photon emission computed tomography imaging was demonstrated and the possibility of imaging-guided drug delivery based on multifunctional PAMAM will be studied in the future.

Introduction

Nuclear medicine relies on collaboration of radiopharmaceuticals and nuclear medicine technologies. Substantial effort has been focused on the development of molecular probes to achieve tumor-specific therapeutic and diagnostic agents (1). Antibody, sugar, folic acid (FA), transferrin, epidermal growth factor and Arg-Gly-Asp peptide are tumor targeting ligands used to give an active targeting-ability to drug carriers (2,3). Of these ligands, FA has attracted wide attention because of its high stability, low-cost, nonimmunogenic character, low molecular weight, and simple chemical

modification. Moreover, a number of tumor cell exhibit high levels of the folate receptors (FRs), and its expression is restricted in FR-negative tissues (4,5). These preferable properties of FA recommend it as a good candidate of targeting groups. Among various folate-probes, one typical case is micromolecule with single target (6-8). The other part is macromolecule-based multimer (9-11).

An important aspect for successful development of the multimeric folate derivatives is to get a suitable spacer and bifunctional chelator (BFC). Polyamidoamine (PAMAM) dendrimer offer such a carrier system, which has a unique branching structure like a tree (12,13). Based on PAMAM dendrimer, an approach to increase the targeting and labeling efficiency has been focused on the construction with more targeting and chelating agents. PAMAM dendrimer has been used for a variety of biomedical applications due to its favourable biocompatibility, controllable properties and carrying capacity (14-17). There are a large number of surface functional groups on the dendrimer's outer shell, which can be modified with a variety of interesting guest molecules.

Positron emission tomography (PET) and single photon emission computed tomography (SPECT) are sensitive imaging techniques for FR-targeted imaging. They have high sensitivity and the possibility to perform functional imaging (18, 19). Several folate conjugates labeled with $^{67/68}$Ga, ^{111}In, ^{64}Cu, and ^{99m}Tc are under pre-clinical and clinical stages (20-22). To perform radiolabeling, the labeling efficiency of dendrimer was precisely controlled by choosing different BFC agents on the surface of PAMAM dendrimer. In most cases, a hydrazino nicotinic chelator (HYNIC) was attached to the FA to bind ^{99m}Tc using coligands N-tris-(hydroxymethyl)-methylglycine (tricine) and trisodium triphenylphosphine-3,3',3''-trisulfonate (TPPTS) (23). HYNIC is a very efficient BFC for ^{99m}Tc labeling at a relatively low concentration. ^{99m}Tc ($t_{1/2}$ =6 h) is a suitable radionuclide for SPECT imaging due to its ideal physical decay characteristics, low cost and easy availability from a

^{99}Mo-^{99m}Tc generator system (24-26). Efficient labeling process and high specific activity come from the perfect match of HYNIC and ^{99m}Tc.

Herein, we described the synthesis of ^{99m}Tc-labeled folate-PAMAM dendrimer for FR targeting and evaluated the effects of the probe properties on diagnostic efficacy. Further, the possibility of imaging-guided drug delivery based on multifunctional PAMAM dendrimer will be studied in the future.

Materials and methods

Chemicals and equipments

Generation-3 (G3) PAMAM dendrimer was purchased from Weihai CY Dendrimer Technology Co., Ltd. (China). G3 PAMAM dendrimer has molecular weight of 6909 Da and 32 primary surface amine groups. N-Hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), tricine, TPPTS and other reagents were purchased from J&K Chemical Ltd. All other reagents and solvents were purchased from commercial suppliers and used without further purification. The eluent $Na^{99m}TcO_4$ were obtained from Zhongshan Hospital Affiliated of Xiamen University. Instant thin-layer chromatography silica gel strips (ITLC-SG) were purchased from Pall Life Sciences. The radioactivity counts were measured with CRC-25R Dose Calibrators (CAPINTEC. INC, USA). The ITLC strips were detected with Mini-Scan radio-TLC Scanner (BioScan, USA). Imaging study was performed by a nanoScan-SPECT/CT preclinical imager (Mediso, HUNGARY). The UV spectroscopy was measured by using microplate spectrophotometer (Multiskan GO, Thermo Fisher,

USA). ^{1}HNMR spectra were measured on a Bruker (600 MHz) spectrometer. Chemical shifts are reported in δ (ppm) values.

Preparation of HP_3FA

Synthesis procedures to obtain HP_3FA can be classified into three main steps. FA-NHS and HYNIC-NHS were synthesized according to the procedure similar to that reported by Guo et al. (27, 28).

HYNIC-NHS was prepared as follows: 22 mL 85% hydrazine hydrate containing 1.5 g 6-chloronicotinic acid was heated at 100 °C for 6 h. Then the reaction mixture was concentrated to afford a white solid. The solid was dissolved in 20 mL water and the pH was adjusted to 5.5 by addition of HCl until a yellow precipitate was formed. The precipitate was collected by filtration, washed with 95% ethanol and ether and dried under high vacuum to afford 1.17 g of 6-hydrazinopyridine-3-carboxylic acid. Then 332 mg 6-hydrazinopyridine-3-carboxylic acid was dissolved in 10 mL dimethyl sulphoxide (DMSO) containing 350 mg 4-dimethylaminobenzaldehyde. The reaction mixture was stirred for 8 h at room temperature (RT). Then 374 mg NHS and 670 mg DCC was added into the flask and the stirring was continued for 18 h. After reaction, the mixture was filtered to give a reddish-brown filtrate containing HYNIC-NHS.

FA-NHS was prepared as follows: In brief, 455 mg DCC and 254 mg NHS were added into 10 mL DMSO containing 650 mg FA. The mixture was stirred at RT for 24 h in the dark. The byproduct was removed by filtration to give a reddish-brown filtrate containing FA-NHS.

HP_3FA was prepared as follows: Filtrate containing HYNIC-NHS was added slowly into 10 mL DMSO containing 1 g PAMAM (G3). The mixture was stirred at RT for 3 d. After that, filtrate containing FA-NHS was added slowly into the mixture and the reaction was continued for another 3 d at RT in the dark. To remove unreacted FA-NHS and HYNIC-NHS, the reaction solution was dialyzed (cellulose membrane with 3.5 k MWCO) against DMSO for 3 d. Then the canary yellow solution in dialysis bag was dropped into ether. The precipitate was collected by filtration, then dried under vacuum to afford the crude product.

Radiolabeling of the compound with ^{99m}Tc

The formulation and route for radiolabeling was shown in **Figure 2**A. In brief, 500 μL tricine solution (80 mg/mL in phosphate-buffered saline (PBS), pH 8), 200 μL HP_3FA solution (1 mg/mL in saline, 10 μL 1 M NaOH), 200 μL TPPTS (5 mg/mL in PBS, pH 8), 20 μL $SnCl_2$ solution (2 mg/mL in 1 M HCl) and 370 MBq $^{99m}TcO_4^-$ were added to a 10 mL vial. The vial was oscillated at 100 °C for 15 min in a lead-shielded water bath and then cooled to RT. ITLC on silica gel was carried out by running a sample on a 10 cm strip using ACD (acid-citrate-dextrose buffer, 0.068 mol/L citrate, 0.074 mol/L glucose, pH 5.0) as mobile phase. The radiochemical purity and the labeling efficiency were analysed using MiniScan radio-TLC. For *in vitro* stability test, ^{99m}Tc-HP_3FA was incubated in saline and serum at 37 °C for 8 h. The stability was monitored using ITLC/ACD system.

Octanol/water partition coefficient

The partition coefficient of ^{99m}Tc-HP_3FA was measured by using the method reported by Guo et al. (27, 28). In brief, 100 μL radiotracer solution was mixed with 2.9 mL of PBS (0.05 mol/L, pH=7.4) and 3 mL of 1-octanol in a micro centrifuge tube. The tube was shocked for 5 min and

centrifuged at 8000 rpm for 5 min. The counts in 100 μL aliquots of both organic and inorganic layers were counted using γ-counter. Experiments were performed with triplicate samples. The partition coefficient value (expressed as log P) was calculated using the following equation: P = (activity in octanol phase-background activity)/(activity in aqueous phase-background activity).

Cell Uptake Study

The cell uptake study described below is based on a published procedure (27, 28). 24 h before the study, the KB cells (a human epithelial carcinoma cell line) were seeded in 24-well plates (5×10^5 cells/well, using FA-free RPMI medium) and incubated at 37 °C. In the study, ^{99m}Tc-HP_3FA (11.1 kBq per 100 μL FFRPMI medium) was added to each well and incubated at 37 °C for increasing periods of time. After incubation, the medium was removed and the cells were gently washed three times with cold PBS (pH 7.4, including 0.2% bovine serum albumin) to determine total radiofolate uptake. Cellular internalization of the ^{99m}Tc-HP_3FA was assessed by additionally washing with 500 μL stripping buffer (0.82 g NaAc, 2.25 g NaCl, 0.5 g bovine serum albumin, 250 mL H_2O, adjust the pH to 4 with acetic acid) two times. The stripping buffer was then transferred to tubes. Finally, the cells were lysed by treatment with 1 M NaOH for 5-10 min and transferred to tubes. The blocking studies were performed by addition of free FA solution (10 μL, 1 mg/mL). Then, 100 μL radiotracer was added, and the well plates were incubated at 37 °C for 1 h, 2 h and 4 h, respectively. After incubation, the medium were removed, and the cells were rinsed with cold PBS. Finally, the cells were lysed by treatment with 1 M NaOH for 5-10 min and transferred to tubes. Each sample was counted for radioactivity using a γ-counter. The cell uptake was normalized in terms of the total added radioactivity (% of total activity).

SPECT Imaging

The experimental procedures and the animal use and care protocols were approved by the Institutional Animal Care and Use Committee of Xiamen University. All experimental protocols were carried out in accordance with the relevant guidelines.

The possibility of FR-targeted SPECT imaging was investigated after radiotracer (18.5 MBq/100 μL) was injected intravenously through tail vein of each mouse. The SPECT imaging was performed with a nanoScan-SPECT/CT preclinical scanner equipped with low energy high-resolution collimators. Anesthesia was induced with isoflurane and spontaneous breathing was maintained during the scan procedure. Computed tomography (CT) data were acquired using an X-ray voltage biased to 50 kVp with a 670 μA anode current, and the projections were 720°. The acquiring parameters of SPECT imaging were as follows: energy peak of 140.5 keV for ^{99m}Tc, window width of 20%, matrix of 256 × 256, medium zoom, and frame: 45 s. The uptakes in different tissues were calculated by drawing regions of interests (ROIs) on the SPECT/CT images.

Results

Chemistry

The synthesis routes and chemical structure of HP_3FA are shown in Scheme S1 and Figure S1A (in supporting information), respectively. To attach HYNIC and FA to PAMAM dendrimer (G3), carboxyl groups of the HYNIC and FA were activated by DCC and NHS. The reaction occurs mainly at the γ-carboxylic group due to it has higher reactivity than that of α-carboxylic group of folate (4,

27, 28). The structure and size of end-product HP_3FA were characterized by UV-spectroscopy (Figure 1A), ^1H NMR (Figure 1B) and dynamic laser scattering (DLS, Figure 1C). The FA has an absorption peak at the wavelength 296 nm, and the absorption at 368 nm is relatively weak. The absorption peak of HYNIC is at 380 nm. We measured the UV absorbance of the tracer HP_3FA. The HP_3FA has two characteristic absorptions at 286 nm and 365 nm. The result suggested that we had successfully synthesized the HP_3FA. Furthermore, the absorbance values of FA and HYNIC in UV spectrophotometer were used to quantify the number of FA and HYNIC per PAMAM. Average of 6.5 FA and 7.3 HYNIC moieties per PAMAM were calculated by plotting the standard curve of FA and HYNIC. The structure of HP_3FA was confirmed by ^1H NMR in DMSO to verify functional groups. The characterization of FA, HYNIC and dendrimer by ^1H NMR spectrum also indicated the successful synthesis of HP_3FA. In DMSO, protons from FA found at 4.0-4.5 ppm and protons on the HYNIC at 7.0-7.5 ppm were found in the ^1H NMR spectrum of final product. By comparing the integration of chemical shifts of the compounds, we speculated that the HYNIC and FA molecules were conjugated to the dendrimer platform. The conjugation of FA and HYNIC groups on PAMAM could also be confirmed by ^1H-NMR of HP_3FA (Figure S1B). FA/HYNIC ratio (about 0.91) could be obtained according to the integral ratios of FA and HYNIC protons (4.0-4.5 ppm for FA; 2.8-3.0 and 7.8-8.0 ppm for HYNIC) to the multiple methylene protons of PAMAM ($-CH_2CO-$, at 2.5-2.8 ppm). Approximate 7.3 FA and 8.0 HYNIC molecules were calculated on each PAMAM dendrimer. This NMR based results are slightly larger than that of UV spectroscopy. Furthermore, the average molecular weight of HP_3FA could be calculated as about 12125 Da. According to the DLS (dynamic laser scattering) results, the size of HP_3FA was about 1.27 nm.

Radiolabeling

^{99m}Tc-HP$_3$FA could be prepared via HYNIC with high labeling efficiency and good stability. Both of labeling efficiency and radiochemical purity of ^{99m}Tc-HP$_3$FA are greater than 95% by ITLC method (Figure 2B). ^{99m}Tc-HP$_3$FA remained stable over 8 h when incubated both in serum and saline (Figure S2 in supporting information). The logP of ^{99m}Tc-HP$_3$FA is -1.70±0.21 indicating the hydrophilicity of the labeled complex. The specific activity of ^{99m}Tc-HP$_3$FA was calculated as ~ 22 GBq/μmol at the end of synthesis.

Cell uptake study

In cell uptake study, as further proof of specific binding of the ^{99m}Tc-HP$_3$FA to FRs, FR-negative 7721 cell line and FR-positive KB cell line were used and incubated from 30 min to 4 h. The ^{99m}Tc-HP$_3$FA conjugate shows high uptakes in the FR-abundant KB cells (Figure 3A). While the 7721 cells which served as control group substantially had no uptakes (Figure 3B). At 1 h after incubation, maximum total binding ratio (27%) of ^{99m}Tc-HP$_3$FA radioactivity was observed on KB cell line. As the surface binding curve showed, ^{99m}Tc-HP$_3$FA can be specifically binding to KB cells via the FRs on the surface of cells. Moreover, the competitive blocked effect of free FA was examined on FR-positive KB cells, which clearly demonstrated that ^{99m}Tc-HP$_3$FA can target cancer cells through FR-mediated effect.

SPECT imaging

Firstly, SPECT imaging of healthy BALB/c mice with ^{99m}Tc-HP$_3$FA were performed to visualize the distribution of the radiotracer in normal mice (shown in Figure S3). The radiotracer was observed

mostly in FR-abundant kidney. In the other organs, such as liver and lung, the uptake of the conjugate kept at low level. This is a common feature of most folate conjugates.

The distribution of the radiotracer ^{99m}Tc-HP$_3$FA was further confirmed by using KB-tumor bearing nude mice in SPECT imaging study. The sagittal, coronal, and transaxial sections of SPECT/CT images were shown in Figure 4, the accumulation of ^{99m}Tc-HP$_3$FA conjugates is visible in kidneys and the KB tumor xenografts localized on both shoulders. The tumor focus was also confirmed through CT scan. Mutually matches relations existed between the SPECT and CT images. More clearly three-dimensional SPECT/CT images were also shown in Figure 4. The results of micro-SPECT/CT imaging showed that ^{99m}Tc-HP$_3$FA conjugate exhibited high uptakes in KB tumor and kidneys. And the tumor-to-muscle and tumor-to-kidney ratios were approximately 25.78 and 0.78 (Table S1), respectively. For the blocking group, we used the FA as inhibitor. The sagittal, coronal, and transaxial sections of SPECT/CT imaging showed that the KB tumor and kidney uptakes of the ^{99m}Tc-HP$_3$FA conjugates were obviously inhibited. The ratios of tumor-to-muscle and tumor-to-kidney were decreased obviously to 1.31 and 0.25, respectively. FR-mediated uptake and renal excretion pathway lead to high radioactivity accumulation in the kidney. Injection of excess FA resulted in an almost complete blockade of the FR-specific tumor and kidney uptake, while non-FR-mediated renal excretion can't be reduced by injecting excess FA. For this reason, in the presence of FA, the tumor-to-kidney ratio was decreased from 0.78 to 0.25 (Table S1).

Discussion

PAMAM dendrimer has drawn great attention and often used as platform to deliver therapeutics and imaging agents (3, 29, 30). As large number of primary amino groups of on the surface of dendrimer, the end groups of PAMAM have high chemical activity and can be modified with many functional molecules, such as therapeutic drugs, targeting groups and imaging agents (31-33). With the development of PAMAM, the conception of the "extensible and modular platform" has become more clearly and come into our research field. To investigate the concept and achieve ideal targeting and imaging results, we chose FA and HYNIC as the candidates in our study.

FR targeting plays a very important role in tumor detection with the assistance of nuclear medicine imaging. As reported in previous studies, to meet the imaging need, some radiolabeled FA derivatives based on dendrimer were synthesized (17, 29, 34, 35). As we know, radiolabeled low molecular weight folate conjugates offer advantages of high tumor to background ratio and faster systemic blood clearance kinetics. Unfortunately, it is a difficult challenge for macromolecule-based folate conjugates. Some studies have demonstrated that FA conjugated to PAMAM dendrimer could target the FR. While, some radiolabeled PAMAM conjugates have the disadvantages of multi-step synthesis, low radiochemical yields and time-consuming purification process, which are not suitable for large-scale productions as needed for clinical application. Moreover, due to the slow clearance of radioactivity from liver, spleen, blood or other non-target tissues, undesired tumor-to-background contrast was achieved, which affected the tumor visualization (34). In our study, the HYNIC could likely improve the pharmacokinetic profiles by increasing labeling efficiency and chelating capability at low HYNIC-conjugate concentrations. Fortunately, the ^{99m}Tc-HP$_3$FA has excellent tumor targeting ability with minimum uptake by the liver, lung and other organs, implying that it has great potential

not only for targeting the primary cancer site but also metastatic sites. As the *in vitro* experiment and imaging study have already reflected the good properties of ^{99m}Tc-HP$_3$FA, biodistribution study was not performed in our study. A direct comparison between ^{99m}Tc-HP$_3$FA and other radiolabeled folate conjugates using percentage of injected dose per gram (%ID/g) was not offered.

Different generation dendrimers show different *in vivo* characteristics. Low generation dendrimer can find tumor quickly and be cleared from the bloodstream rapidly. High generation PAMAM are more likely to accumulate in liver while low generation PAMAM tend to localize in kidney. There have more active-sites on the surface of higher generation dendrimer which could be modified with functional group (3, 30). In this study, PAMAM (G3) can provide sufficient primary amino groups to attach HYNIC and FA, but also has excellent pharmacokinetic profiles. It is particularly gratifying that based on the unique structural characteristics of PAMAM dendrimer, by replacing HYNIC or FA with other corresponding functional groups, this versatile strategy can be readily extended to other sensitive multimodal imaging and/or enhanced therapy of different cancers. In other words, it is feasible to synthesize mono-, di-, and even multi-functionalized conjugates using PAMAM dendrimer as platform with facile modification methods. By different components' assembly, targeting groups, therapeutic elements, and imaging reagents were mixed with PAMAM dendrimer platform simultaneously or sequentially to give mono-, di-, and multi-functional conjugates for different uses (33, 36, 37). These achievements have shown brilliant future to the final implementation of "extensible and modular platform". Further studies are under way to implement these ideas by using more sophisticated functional groups basing on more efficient synthetic scheme. We anticipate these attempts will facilitate further advancement of multifunctional PAMAM.

Conclusion

A new radio-compound of ^{99m}Tc-HP$_3$FA was synthesized successfully with specifically FR targeting ability. It shows promising characteristics in comparable with many FA-modified PAMAM dendrimers. This new delivery system overcomes the obstacles of the non-specific uptake in liver and lung. The feasibility of ^{99m}Tc-HP$_3$FA for early diagnosis of FR-positive tumors with noninvasive SPECT imaging was demonstrated in this study. The possibility of imaging-guided drug delivery or multimodal imaging system based on this versatile strategy will be studied in the follow-up experiment.

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Conflicts of Interest: The authors declare no conflict of interest.

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The legends of tables and figures:

Figure 1. (A) The UV spectroscopy of HYNIC, FA and HP_3FA. (B) ^{1}H-NMR spectrums of FA, HYNIC, PAMAM and HP_3FA. (C) The average size for HP_3FA measured by DLS was 1.27 nm.

Figure 2. (A) Radiolabeling procedure of ^{99m}Tc-HP_3FA. (B) The radiolabeling efficiency and radiochemical purity of ^{99m}Tc-HP_3FA were tested by ITLC-SG/ACD. ^{99m}Tc-HP_3FA stayed at the point of origin (R_f: 0-0.1), whereas hydrolyzed ^{99m}Tc and $^{99m}TcO_4^-$ moved to the front (R_f: 0.8-1.0).

Figure 3. The cell binding properties of ^{99m}Tc-HP_3FA in the KB cells (A) and the 7721 cells (B). The cell binding (cell total binding, internalization and blocked by excess FA, $P < 0.05$) was expressed as percentage of total added radioactivity.

Figure 4. (A and B) The SPECT/CT imaging of the KB-tumor bearing nude mice at 2 h. (C and D) Blocking imaging study performed by received 100 μg FA 10 min prior to the injection of ^{99m}Tc-HP$_3$FA.