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Synthesis, Radiolabelling and *in vitro* Stability Study of $^{99m}Tc(CO)_3^+$ Labeled Dendrimer PAMAM-Folic Acid Conjugate

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Dendrimer polyamidoamine generation five-folic acid conjugate was synthesesed and radiolabelled with *fac*-$[^{99m}Tc(CO)_3(H_2O)_3]^+$, its *in vitro* stability was evaluated further. Both of the labeling yield and radiochemical purity of the G5-FA-DTPA-$^{99m}Tc(CO)_3$ conjugate exceeded 95%. More than 95.7% and 93.1% of the conjugate still keeps its original structure in PBS and new-born calf serum solution respectively.

Keywords dendrimer, PAMAM, $^{99m}Tc(CO)_3^+$, radiolabelled, folic acid

Introduction

Dendrimers represent a unique class of nanostructures, playing an important role in the field of nanobiotechnology.[1-4] Poly(amidoamine) (PAMAM) was one of the most studied dendrimers, which was first synthesized and developed in Dow Laboratories between 1979 and 1985.[5,6] Dendrimers are synthesized from branched monomer units in a step-wise manner, thus it is possible to precisely control their molecular properties, such as size, shape, dimension, density, polarity, flexibility and solubility, by choosing different building/branching units and surface functional groups.[7] The large numbers of surface functional groups on dendrimer's outer shell can be modified or conjugated with a variety of interesting guest molecules.[8] Over the last several years, increasing interest has been attracted to the application of dendrimers as targeting carriers in cancer therapy and imaging.[9-11] Recent studies have demonstrated that the conjugation of special targeting moieties to dendrimers can lead to preferential distribution of the cargo in the targeted tumor-cells. The Baker's group has investigated several variations of folic acid-conjugated dendrimers for targeted drug delivery.[12-15] A doubly cyclized RGD (RGD-4C) peptide and Alexa Fluor 488 fluorescent label were conjugated to partially acetylated G5-PAMAM for the targeting of tumor neovasculature via uniquely expressed $\alpha_v\beta_3$ integrins.[16] In the Yang's study, biotin and fluorescein were conjugated to partially acetylated G5-PAMAM and examined *in vitro* against HeLa cells.[17] However the fluorescein labeled dendrimer conjugation is very difficult to be detected in live animal image study. So radiolabeled dendrimer conjugates will find wide range of applications in SPECT or PET image (SPECT: Single Photon Emission Computed Tomography; PET: Positron Emission Computed Tomography). Single-photon-emitting radionuclides have many advantages in the applications.[18] The radioisotope used herein, ^{99m}Tc, is present in almost all particle-based radiopharmaceuticals including Technescan and Microlite and is currently the most used imaging agent in nuclear medicine due to its ideal physical properties ($t_{1/2}$=6 h, E =140 keV, 89% abundance), suitable dosimetry (low radiation dose) and high specific activity. It is generator-produced (^{99}Mo/ ^{99m}Tc) and available at a reasonable cost. The most recent labeling precursor to yield ^{99m}Tc radiopharmaceuticals is the $[^{99m}Tc(CO)_3(H_2O)_3]^+$ complex cation. This complex is suitable for coordination to a wide variety of ligands, its three potential binding sites are readily available for coordination, since the aqua ligands are labile and easily replaced.[19,20] In this paper the 5th generation PAMAM dendrimer-folic acid conjugate (G5-FA) was synthesesed, characterized and radiolabelled with $^{99m}Tc(CO)_3^+$. To the best of our knowledge, this is the first time to use $^{99m}Tc(CO)_3^+$ labeling the dendrimer folic acid conjugate as potential SPECT imaging agent.

Methods

Materials

1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide HCl (EDC, 98%), polyamidoamine (PAMAM) dendrimer generation 5 (G5), and folic acid (FA) were purchased

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from Aldrich Co., Ltd. Anhydrous sodium carbonate, potassium sodium tartrate tetrahydrate, sodium borohydride, dimethyl sulfoxide (DMSO, 99%), dimethylformamide (DMF, 99%), and dialysis membrane (MWCO, 3500 and 14000) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai). Carrier-free ^{99m}Tc-perrhenate was freshly eluted with saline from ^{99}Mo/^{99m}Tc-generator (Shanghai Yuanpu Isotope Technology. Co., Ltd.), 2-(*p*-isothiocyanatobenzyl)-6-methyl-diethylenetriaminepentaacetic acid (1B4M-DTPA) was a gift from Dr. Martin W. Brechbiel (NIH). All of the reagents were used as received without further purification. ^{1}H NMR spectra were performed in D_2O solution on Bruker AVANCE DRX 500 spectrometer.

Reverse phase high performance liquid chromatography

A Phenomenex (Torrance, CA) Jupiter C5 silica based HPLC column (250—4.6 mm, 300 Å) was used for the separation of analyst. Two Phenomenex Widepore C5 guard columns (4—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 at a flow rate of 1 mL/min, reaching 50∶50 after 30 min. Trifluoroacetic acid was at 0.14 wt% concentration in water. The conjugates were dissolved in the mixture (90∶10 water/acetonitrile). The injection volume in each case was 200 μL.

Preparation of dendrimer PAMAM generation 5-folic acid-DTPA conjugate (G5-FA-DTPA)

The folate-conjugated dendrimer-chelate was prepared by coupling folate to the PAMAM via a carbodiimide reaction, followed by reaction of the remaining free amines with 1B4M-DTPA. Briefly, 12.2 mg of FA (MW=441.4 g/mol) reacted with 74.2 mg of EDC (MW=191.71 g/mol) in a mixture of 6 mL of dry DMF and 2 mL of dry DMSO under a nitrogen atmosphere for 1 h. This organic reaction mixture was added dropwise to the DI water solution (15 mL) of 100 mg (MW=28824 g/mol) of G5-PAMAM. The reaction mixture was vigorously stirred for 3 d. After dialysis (using PBS buffer and DI water 3 times and 4 L, respectively) and lyophilization, the yield was 93.7%. The G5-FA was concentrated to 5 mg/mL and reacted with a 128-fold molar excess of 1B4M-DTPA (MW=555 g/mol) at 40 ℃. The reaction was maintained at pH=9—10 with 1 mol/L NaOH for 24 h. Another additional equal amount of 1B4M-DTPA was added to the mixture after 24 h as a solid, and the pH was maintained as before for another 24 h. The resulting preparation was purified by dialysis (using PBS buffer and DI water three times and 4 L, respectively) and lyophilization, repeated 2 times, yield 97.4%.

Preparation of radioactive intermediate $^{99m}Tc(CO)_3(H_2O)_3^+$

The $^{99m}Tc(CO)_3(H_2O)_3^+$ was prepared by adding 2 mL of $^{99m}TcO_4^-$ from a commercial generator (5 mCi) to a 10 mL vial containing potassium boranocarbonate (20 mg), sodium potassium tartrate tetrahydrate (6.6 mg), and potassium tetraborate pentahydrate (5.4 mg) and 101 kPa carbon monoxide (headspace volume). The reaction mixture was heated for 25 min in boiling water at 101 kPa or in a carbonyl reaction kit viral. Before the reaction was started the pH was adjusted to 11.5 by adding 7.6 mg KBH_4, 13.5 mg *L*-tartaric acid, 20 mg lactose monohydrate, 10 mg $K_2B_2O_{24}$•H_2O to the solution, then lyophilization and sealed under CO atmosphere. The mixture was then shaken vigorously for 30 s, heated for 10 min at 100 ℃, cooled to room temperature, and pH was adjusted to 7 with 1 mol•L^{-1} hydrochloric acid. $^{99m}Tc(CO)_3(H_2O)_3^+$ was successfully prepared with high radio-yields (>95%). The solution was analyzed by radio-TLC.[21-23]

$^{99m}Tc(CO)_3(H_2O)_3^+$ labeled G5-FA-DTPA conjugate

To a 10 mL serum vial 1 mL of the aqueous $^{99m}Tc(CO)_3(H_2O)_3^+$ solution was added, followed by 25 mg G5-FA-1B4M. Then the mixture was heated at 75 ℃ for 30 min. The solution was analyzed by radio-TLC and RP-HPLC.

In vitro stability study

The stability of the labeled G5-FA-DTPA was determined by challenging with phosphate buffer saline (PBS, pH 7.4) and new-born calf serum respectively. The mixture was incubated at 37 ℃ up to 6 h. The radiochemical purity of $^{99m}Tc(CO)_3$-G5-FA-DTPA was evaluated using RP-HPLC.

Results and discussion

Folic acid and DTPA was conjugated to 5.0 G PAMAM dendrimer

Conjugation of folic acid (FA) to the dendrimer was carried out via condensation between the γ-carboxyl group of folic acid (FA) and the primary amino groups of the dendrimer. The active ester of FA, formed by reaction with EDC in DMSO-DMF (1∶3 solvent mixtures), was added dropwise to a solution of DI water containing G5-PAMAM and was vigorously stirred for 3 d to allow for the FA to be conjugated to the G5-PAMAM completely. FA has two different kinds of carboxyl groups. It is obvious that the α-carboxyl group may participate in the condensation reaction, but its reactivity is much lower, as compared to the γ-carboxyl group. In this reaction the γ-carboxylic group possesses a higher reactivity during carboiimide-mediated coupling to amino groups as compared to that of the α-carboxyl group. When the γ-carboxylic group on FA is used for conjugation to the dendrimer, FA retains a strong affinity toward its receptor, allowing the FA moiety of the conjugate to retain its ability to act as a targeting agent.[12,24] The number of FA in one G5-PAMAM was measured by ^{1}H NMR, using the ^{1}H

proton integration method. On the basis of the integration values of the methylene protons of $CH_2C(O)$ in G5 PAMAM dendrimer and the aromatic protons in the FA, the number of attached FA molecules was calculated to be 6.9. G5-FA (D_2O): Aliphatic peaks at δ 2.33, 2.54, 2.73, 3.20 correspond to protons in G5 PAMAM dendrimer. The folate conjugates were also determined by UV spectroscopy. The UV spectrum for G5-FA conjugate was presented in Figure 1, showing the defined peak for FA at 282 and 348 nm, which were not found in the UV spectra for G5-PAMAM.

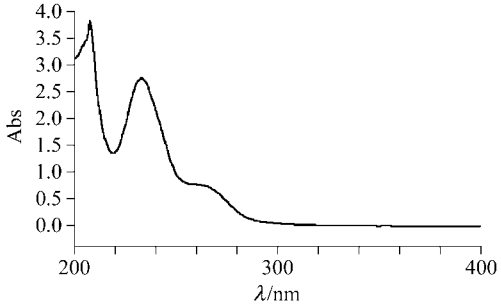

Figure 1 UV spectrum for G5-FA.

1B4M-DTPA is an ideal bifunctional chelating agent. That is because the isothiocyanates is very easy to react with the primary amines on the surface of dendrimer PAMAM in which the degree of functionality can be easily controlled by the ratio of the starting materials. The number of 1B4M-DTPA molecules was also determined by ^1H NMR. As the same above, average 105 1B4M-DTPA molecules are present on the surface of each G5-FA dendrimer.

Preparation of the $^{99m}Tc(CO)_3(H_2O)_3^+$ and label G5-FA-DTPA

In all preparations, the radiochemical purity of the $^{99m}Tc(CO)_3(H_2O)_3^+$ intermediate exceeded 95% determined by radio-TLC on Whatman No.1 paper strips eluted with acetonitrile. The chromatography strips were cut into 2 cm sections, beginning at the origin and continuing to the solvent, and the ^{99m}Tc activity was scanned on an automatic TLC linear analyzer. The R_f value of $^{99m}Tc(CO)_3(H_2O)_3^+$ is 0—0.1. This intermediate was used without further purification (Figure 2).

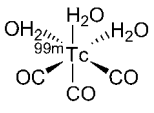

Figure 2 Chemical structure of $^{99m}Tc(CO)_3(H_2O)_3^+$.

Preparation of $^{99m}Tc(CO)_3$-G5-FA-DTPA is in the aqueous $^{99m}Tc(CO)_3(H_2O)_3^+$ solution fon about 30 min at 75 ℃. The ligand concentrations in the reactions were in 10^{-6}—10^{-7} mol/L range. The formation of the ^{99m}Tc tricarbonyl complexes was monitored by radio-TLC. The labeling yields and radiochemical purity of the G5-FA-DTPA-$^{99m}Tc(CO)_3$ product also exceeded 95% as determined by RP-HPLC. The retention time is 4.36 min (Figure 4). The exact chemical speciation of the $[^{99m}Tc]Tc(CO)_3$-G5-FA-$DTPA_{105}$ product has not been defined. It is presumed to bind the "$[^{99m}Tc]Tc(CO)_3^+$" fragment as a tridentate ligand. It is possible having three ways for DTPA to bind the $[^{99m}Tc]Tc(CO)_3^+$ using the terminal amine nitrogen and the two associated acetate carboxyl oxygen atoms (Figure 3), analogous to the coordination of *fac*-$Tc(CO)_3^+$ by iminodiacetic acid, via the three amine N atoms of the DTPA backbone as well as by two amine N atoms and one carboxyl O donor to satisfy the requirements of the Tc(I) center. In aqueous solution, all of these isomeric species may exist in equilibrium and none can be excluded by the present study. However DTPA is not an ideal bifunctional chelating agent for labeling bioactive compound with ^{99m}Tc-tricarbonyl core. Why could we get high labeling yields and radiochemical purity? We believe the amine group on the surface of the PAMAM was involved in the coordination.

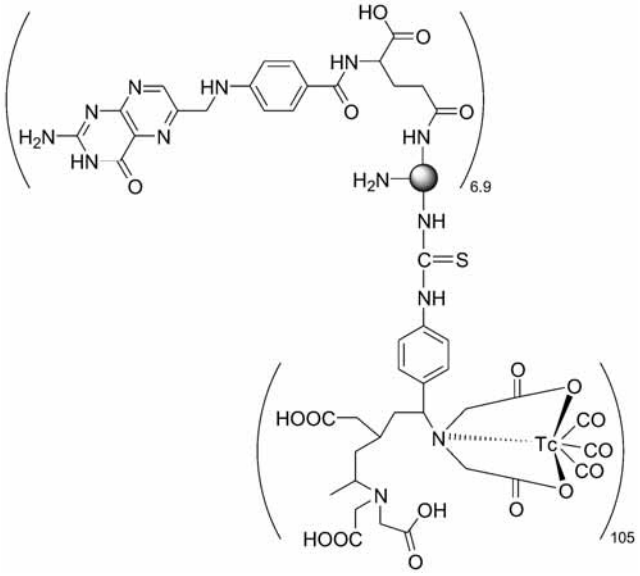

Figure 3 Chemical structure of $[^{99m}Tc]Tc(CO)_3$-G5-FA-DTPA.

Stability of the $^{99m}Tc(CO)_3$ labeled conjugate was evaluated in PBS and serum respectively in period of 6 h at 37 ℃. The result was shown in Figure 5. About 95.7% (in PBS) and 93.1% (in serum) still keeps its original structure after labeling at 37 ℃ within 6 h.

Conclusion

PAMAM dendrimer with folic acid targeting and ^{99m}Tc imaging moieties was successfully synthesized and characterized. The radiolabeling of *fac*-$[^{99m}Tc(CO)_3(H_2O)_3]^+$ with the dendrimer conjugate was straightforward and highly efficient with quantitative yield. The $^{99m}Tc(CO)_3^+$-dendrimer PAMAM G5-FA conjugate demonstrates excellent stability in phosphate buffer saline (pH 7.4) and serum over a period of 6 h after labeling. The detailed bioevaluation and further

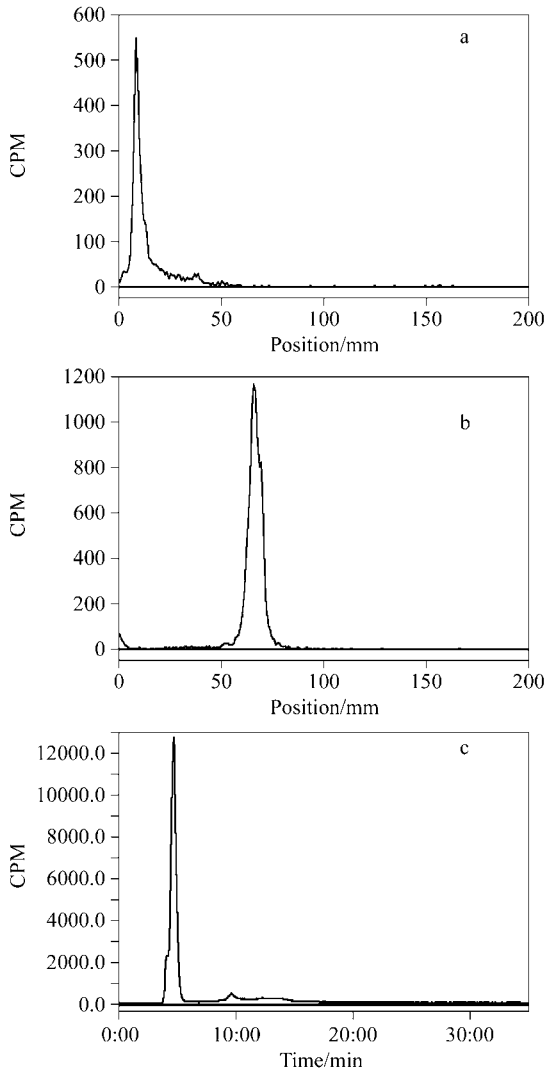

Figure 4 The radio TLC of $^{99m}Tc(CO)_3^+$ (a), G5-FA-DTPA $^{99m}Tc(CO)_3$ (b) and RP-HPLC analyses of $^{99m}Tc(CO)_3^+$ and G5-FA-DTPA-$^{99m}Tc(CO)_3$ (c).

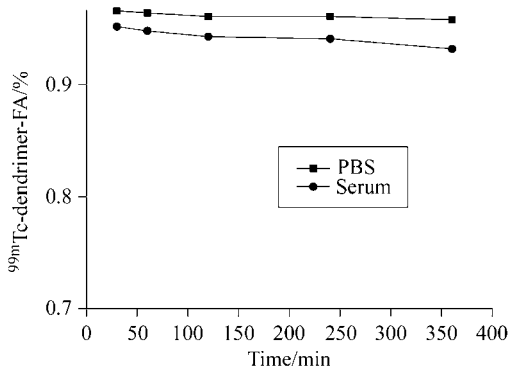

Figure 5 Stability of the $^{99m}Tc(CO)_3(H_2O)_3^+$ labeled conjugate in PBS and serum.

structure modification is under way and will be reported in due course.

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